REMARKS

Entry of the foregoing and further and favorable reconsideration of the subject application, in light of the following remarks, are respectfully requested. By this amendment, claims 6 and 10 have been canceled, without prejudice or disclaimer to the subject matter disclosed therein. Claim 1 has been amended to recite that the linker is a polypeptide linker. Support for this amendment can be found in claim 1 as originally filed. Claim 1 has also been amended to recite that the receptor can be a cytokine receptor or subunit thereof and the ligand can be a cytokine ligand or subunit thereof. Support for this amendment to claim 1 can be found, at the very least, on pages 2 and 3 of the specification as filed. Claims 2 and 3 have been amended merely to clarify the claims. Claim 11 has been amended to recite that either the conjugate itself or a plasmid which expresses the conjugate can be mixed with cells to influence the protein interaction. Support for this amendment can be found, at the very least, in Examples 4 and 5 and on page 5, lines 22-33, of the specification as filed. New claim 12 has been added. Support for new claim 12 can be found, at the very least, on page 5, line 35, to page 6, line 6, of the specification as filed. Finally, the specification has been amended to include reference to SEQ ID Nos. No new matter has been added by the present amendment.

Entry of this amendment is proper under 37 C.F.R. § 1.116 because the amendment places the application in condition for allowance for the reasons discussed herein; does not raise any new issue requiring further search and/or consideration; and places the application in better form for an appeal, should an appeal be necessary. The amendment is necessary and was not earlier presented because the applicants have not previously

responded to the rejections presented by the Examiner. Entry of the amendment is thus respectfully requested.

Rejection of Claims 1-4, 6-9 and 11 Under 35 U.S.C. § 112, First Paragraph

Claims 1-4, 6-9 and 11 have been rejected under 35 U.S.C. § 112, first paragraph, for purportedly not being enabled for a conjugate comprising a cytokine and its ligand that are linked by disulfide bonds. For at least all of the reasons set forth below, withdrawal of this rejection is believed to be in order.

The claims have been amended to recite that the two polypeptides are linked by a polypeptide linker. Applicants of course reserve the right to pursue the canceled subject matter in a continuation application.

In light of these remarks, applicants respectfully request withdrawal of this rejection under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 1-4, 6-9 and 11 Under 35 U.S.C. § 112, Second Paragraph

Claims 1-4, 6-9 and 11 have been rejected under 35 U.S.C. § 112, second paragraph, for purportedly being indefinite. For at least all of the reasons set forth below, withdrawal of this rejection is believed to be in order.

The claims have been amended to overcome each of the rejections under 35 U.S.C. § 112, second paragraph. Specifically, claim 1 has been amended to recite that a polypeptide is the linker (thereby rendering moot the rejections of claim 1). Claims 2 and 3 have been amended (as well as claim 1) to clarify what is meant by "subunit." Claim 11

has been amended to include clearly recited method steps and to clarify how the interaction between proteins is influenced.

In light of these remarks, applicants respectfully request withdrawal of this rejection under 35 U.S.C. § 112, second paragraph.

Rejection of Claims 1-4, 6-9 and 11 Under 35 U.S.C. § 103(a)

Claims 1-4, 6-9 and 11 have been rejected under 35 U.S.C. § 103(a) for purportedly being obvious over Sui et al (*PNAS 92*:2859-2863 (1995)) and further in view of Wong et al (WO 96/04314). For at least all of the reasons set forth below, withdrawal of this rejection is believed to be in order.

Sui et al disclose that the addition of soluble human IL6-receptor (sIL-6R), IL6 and stem cell factor (SCF) to hemopoietic progenitor cells leads to a stimulation of the expansion of these cells. Sui et al does not disclose or suggest a conjugate of a cytokine receptor and a cytokine. Although Sui et al discusses IL-6R/IL-6 complexes, the discussion is regarding complexes that are formed after administration. There is no disclosure by Sui et al of forming a complex between IL-6R and IL-6 prior to administration. See, for example, page 2860, column 1, "Clonal Culture" where it is discussed that various combinations of cytokines, with or without IL-6R, were plated. Sui et al only discloses what was mentioned in the introductory part of the present application (see third paragraph of page 1), i.e. the separate administration of IL6 and IL6-receptor.

Wong et al disclose MHC fusion complexes that contain a MHC molecule with a peptide-binding groove and a presenting peptide covalently linked to the MHC molecule

via a polypeptide linker. These complexes are used to modulate the activity of T cells. Wong et al does not disclose or suggest a complex comprising a cytokine receptor and a cytokine linked by a polypeptide. The purpose of Wong et al is to provide a MHC-peptide complex which can activate T-cells. The complex was formulated so that the MHC molecule and the peptide are positioned such that they can activate the T-cells. The presentation of a MHC molecule complexed with a peptide is completely different from the presentation of a cytokine complexed with a cytokine receptor.

The applicants surprisingly found that with a conjugate according to the present invention, the effects of the individual polypeptides (i.e. the cytokine and cytokine receptor) can be increased dramatically. This is proven by the fact that expansion and colony formation of $CD34^+$ cells can be increased by 300% as compared to the separate addition of IL-6 and IL-6 receptor (see Example 5 in the application as filed).

A conjugate according to the present invention also serves for influencing liver cells so as to stimulate the haptoglobin expression many times over as compared to the separate addition of IL-6 and IL-6 receptor (see Example 4 in the application as filed). By using a conjugate according to the present invention, diseased liver tissue can be regenerated completely, which is not possible with the use of individual polypeptides. Reference is made in this connection to enclosed U.S. Patent No. 5,919,763 (Galun), attached hereto as Exhibit A, and the enclosed documents Galun et al (*FASEB J. 14*:1979-1987 (2000)) (Exhibit B) and Hecht et al (*Mol. Therapy 3*:683-687 (2001)) (Exhibit C).

The fact that references can be combined or modified is not sufficient to establish *prima facie* obviousness. The prior art must suggest the desirability of the combination.

See MPEP Sec. 2143.01. Since Sui et al does not disclose or even suggest the administration or use of a cytokine/cytokine receptor complex, and since the complex disclosed by Wong et al is completely different from that of the present invention (i.e. it does not comprise either a cytokine or a cytokine receptor) there would be no motivation to combine the disclosures of these two references to arrive at the present invention, i.e. a complex comprising a cytokine and a cytokine receptor. Thus, the Examiner has not established a *prima facie* case of obviousness.

Even if there was a motivation to produce a complex comprising a cytokine and a cytokine receptor, there would be no expectation of obtaining the unexpectedly superior properties of such a complex. Evidence of unobvious or unexpected advantageous properties, such as superiority in a property the claimed compound shares with the prior art, can rebut *prima facie* obviousness. See MPEP Sec. 716.02(a). Thus, even if a case of *prima facie* obviousness was established, it is rebutted by the showing of unexpected superiority of the cytokine/cytokine receptor complex as compared to administration of the individual cytokines and cytokine receptors.

In light of these remarks, applicants respectfully request withdrawal of this rejection under 35 U.S.C. § 103(a).

CONCLUSION

From the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order and such action is earnestly solicited.

In the event that there are any questions relating to this application, it would be appreciated if the Examiner would telephone the undersigned attorney concerning such questions so that prosecution of this application may be expedited.

Respectfully submitted,

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Date: May 24, 2002

Attachment to Amendment and Reply dated May 24, 2002

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Page 6, Paragraph Beginning at Line 19

--Fig. 1 shows the amino acid (SEQ ID No:1) sequence (and DNA sequence (SEQ ID No:2)) [sequence] of a fusion polypeptide H-IL-6 according to the invention. Sequences for the restriction enzyme SalI (GTCGAC), the signal peptide (MLAVGCALLAALLAAPGAA) and the linker (RGGGGSGGGGSGGGGSVE) are indicated. The linker links the COOH terminus of human sIL-6R with the NH_2 terminus of human IL-6.--

Page 6, Paragraph Beginning at line 27

--Fig. 2 shows the amino acid (SEQ ID NO:3) sequence (and DNA sequence (SEQ ID NO:4)) [sequence] of a fusion polypeptide H-IL-6 according to the invention. Sequences for the restriction enzyme SalI (GTCGAC), the signal peptide (MLAVGCALLAALLAAPGAA) and the linker (RGGGGSGGGGSVE) and indicated. The linker links the COOH terminus of human sIL-6R with the NH_2 terminus of IL-6.--

Page 6, Paragraph Beginning at line 35

--Fig. 3 shows the amino acid sequence of the IL-6 polypeptide (SEQ ID NO:5) present in a fusion polypeptide H-IL-6 according to the invention.--

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Page 7, Paragraph Beginning at line 19

--The DNA of fig. 1 was prepared. For this purpose, human IL-6R cDNA (Schooltink et al., Biochem. J. (1991) 277, 659-664) was used. This cDNA was cloned into the epression plasmid pCDM8 via restriction site Xho I (Müllberg et al., Eur. J. Immunol. (1993) 23, 473-480). By means of a polymerase chain reaction (PCR), an sIL-6R fragment was generated by using the primer (1) (pCDM8 5' primer: 5' TAATACGACTCACTATAGGG 3' (SEQ ID NO:6)) and primer (2) (sIL-6R 3' primer: 5' CCGCTCGAGCTGGAGGACTCCTGGA 3' (SEQ ID NO:7)) under normal conditions. After being cut with restriction enzymes Hind III and Xho I, this fragment was cloned into the open plasmid pCDM8. The plasmid pCDM8-sIL-6R formed. Thereafter, a second PCR reaction was carried out with IL-6 cDNA which has also been cloned into the expression plasmid pCDM8 by using Xho I. The primers (3) (IL-6-5' primer: 5' CGGCTCGAGCCAGTACCCCCAGGAGAA3' (SEQ ID NO:8)) and primer (4) (pCDM8 3' primer: 5' CCACAGAAGTAAGGTTCCTT3' (SEQ ID NO:9)) were used. The PCR product was cut with restriction enzymes Xho I and Not I and cloned into plasmid pCDM8-sIL-6R. The plasmid pCDM8-sIL-6R-IL-6 formed. Thereafter, a synthetic linker was prepared which consisted of two oligonucleotides: primer (5) (5' TCGAGGAGGTGGAGGTTCTGGAGGTGGAGGTTCTGGAGGTGGAGGTTCTG 3' (SEQ ID NO:10)) and primer (6) (5' TCGACAGAACCTCCACCTCCAGAACCTCCACCTCCAGAACCTCCACCTCC3'

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(SEQ ID NO:11)). Oligonucleotides (5) and (6) were combined according to standard methods into a double strand and then cloned into the plasmid pCDM8-sIL-R-IL-6 digested by the restriction enzyme Xho I. The plasmid pCDM8-H-IL-6 formed.--

Please replace the paragraph beginning on page 8, line 4, with the following:

--The DNA of fig. 2 was prepared. For this purpose, the steps as described in Example 1 were carried out. However, the following primers were used instead of [as] primers (5) and (6), respectively: primer (7) [(5)] (5' TCGAGGAGGTGGAGGTTCTGGAGGTGGAGGTTCTG 3' (SEQ ID NO:12)) and primer (8) [(6)] (5' TCGACAGAACCTCCACCTCCAGAACCTCCACCTCC 3' (SEQ ID NO:13)). The plasmid pCDM8-H-IL-6-(2) was obtained.--

Attachment to Amendment and Reply dated May 24, 2002

Marked-up Claims 1-3 and 11

1. (Twice Amended) A conjugate comprising two polypeptides with a mutual affinity, one polypeptide being a cytokine receptor, or a subunit thereof, and the other polypeptide being a cytokine, or a subunit thereof, as ligand and the polypeptides being linked with each other via a polypeptide linker [, wherein the linker is a disulfide bridge formed by two polypeptides or a polypeptide].

2. (Twice Amended) The conjugate according to claim 1, wherein the receptor is [present in the form of its] a subunit of a cytokine receptor and binds to said [binding the] ligand.

3. (Twice Amended) The conjugate according to claim 1, wherein the cytokine is a subunit of a cytokine and binds to said [ligand is present in the form of its subunit binding the] receptor.

11. (Amended) A method for influencing the interaction between proteins comprising [using] incubating the conjugate according to [claim] any one of claims 1 to 4 [along with the DNA coding for said conjugate] or the expression plasmid according to claim 8 with cells and determining said interaction.

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US005919763A

United States Patent [19]

Galun et al.

[11] Patent Number: 5,919,763

[45] Date of Patent: Jul. 6, 1999

[54] IL-6/SIL-6R COMPLEX FOR PROMOTION OF LIVER FUNCTIONS

[75] Inventors: Eithan Galun, Har Adar, Israel; Stefan Rose-John; Malte Peters, both of Mainz, Germany

[73] Assignee: Hadasit Medical Research Services and Development Company Ltd., Jerusalem, Israel

[21] Appl. No.: 09/087,796

[22] Filed: Jun. 1, 1998

[51] Int. Cl.[6] A61K 38/00

[52] U.S. Cl. 514/12; 514/2; 514/893; 514/894; 530/351; 424/85.1; 424/85.2

[58] Field of Search 424/85.1, 85.2; 514/893, 894, 2, 12; 530/351

[56] References Cited

PUBLICATIONS

Medline on 97187685, Fischer et al., *Nature Biotech.*, 15(2), 142–5. (abstract), Feb. 1997.

Primary Examiner—Cecilia J. Tsang
Assistant Examiner—C. Delacroix-Muirheid
Attorney, Agent, or Firm—Mark M. Friedman

[57] ABSTRACT

A method for treating an injury of a liver of a subject with a composition featuring a pharmaceutically acceptable amount of an IL-6/sIL-6R complex, preferably Hyper-IL-6. The composition is administered to the subject such that the injury to the liver is treated.

7 Claims, 5 Drawing Sheets

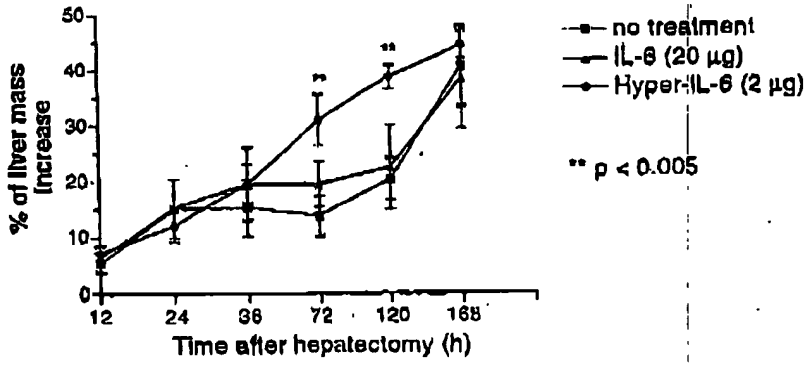

FIGURE 1

Figure 1: *Hyper-IL-6 causes an accelerated reconstitution of the liver weight following partial hepatectomy.*

Immediatedly following a 50% partial hepatectomy, IL-6 (20µg/mouse), or Hyper-IL6 (2 µg/mouse), or physiological saline was injected intraperitoneally into mice. At the time points indicated in the figure, mice were sacrificed, the remnant livers were removed and the percentage of liver weight increase compared to time 0 at hepatectomy was determined (see Methods). Four to six mice were operated at each time point in each treatment group. Mean values ± standard deviations are presented.

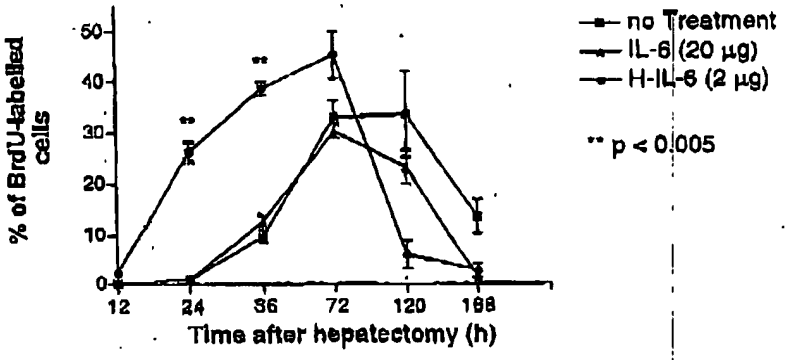

FIGURE 2

Figure 2: *Hyper-IL-6 significantly accelerates liver proliferation in mice following partial hepatectomy in mice.*

Following a 50% partial hepatectomy, IL-6 (20 µg/mouse), or Hyper-IL-6 (2 µg/mouse), or physiological saline was injected intraperitoneally into mice. One hour before the mice were sacrificed, 50 mg/kg body weight BrdU in PBS was injected intraperitoneally into the mice. After removal of the remnant livers, the organs were fixed in 4% formaldehyde and embedded in paraffin. Tissue sections were subjected to BrdU immunhistochemistry. The percentage of BrdU-positive nuclei were counted in at least three mice per treatment group. Mean values ± standard deviation are shown.

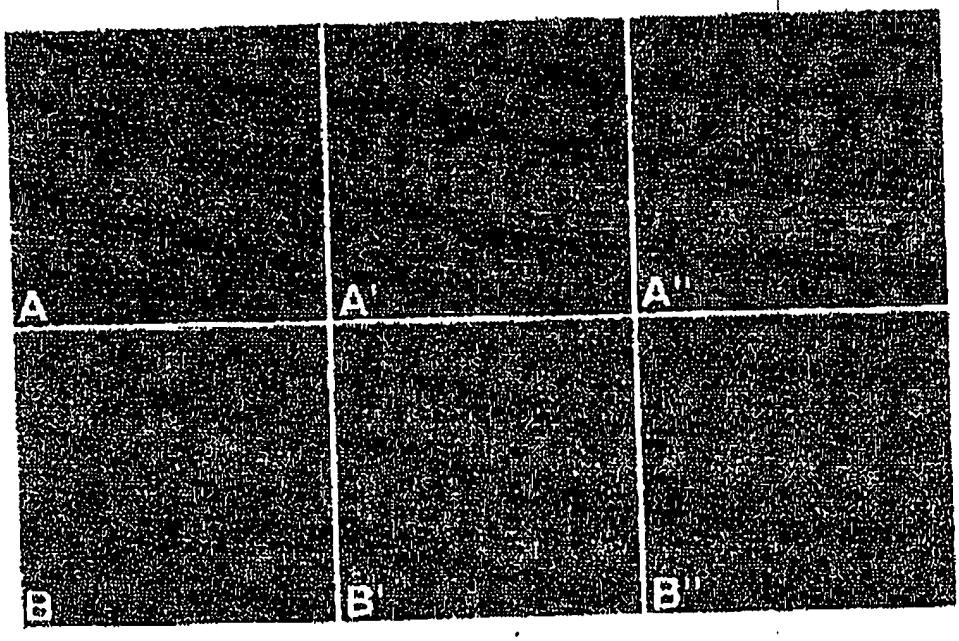

FIGURE 3

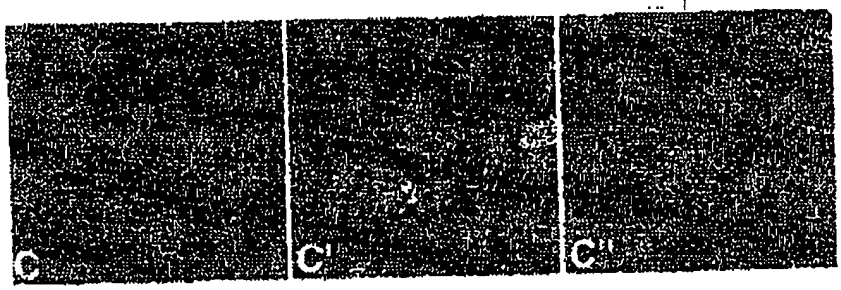

FIGURE 3 (continued)

Figure 3: *BrdU labelling following partial hepatectomy in mice.*

Immunohistochemical detection of BrdU incorporation in S-phase liver nuclei as an indicator of liver cell proliferation. Following 50% partial hepatectomy, mice were either left untreated (A-C), treated with 20 μg IL-6/mouse (A'-C'), or treated with 2 μg Hyper-IL-6 (A''-C''). Mice were sacrificed 24 hours (A, A', A''), 36 hours (B, B', B''), or 120 hours (C, C', C'') following surgery. One hour before the animals were sacrificed 50 mg/kg body weight BrdU in PBS was injected intraperitoneally. The bars represent 100 μm.

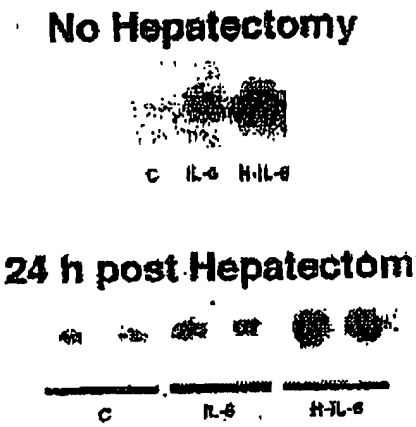

FIGURE 4

Figure 4: The acute phase response is intact in mice following partial hepatectomy.

Upper panel: 24 hours after intraperitoneal injection of saline, 20 μg IL-6 alone, or 2 μg Hyper-IL-6, blood was drawn from the animals which did not undergo partial hepatectomy. One μl of the murine serum was loaded on a 12.5 % SDS gel and was subjected to SDS-PAGE. The gel was blotted onto a nitrocellulose membrane and was subjected to Western blotting using a monoclonal antibody specific for murine haptoglobin.

Lower panel: Mice that had undergone partial hepatectomy were immediately treated with either saline, 20 μg IL-6, or with 2 μg Hyper-IL-6. 24 hours after the operation, blood was drawn from the animals and serum was subjected to Western blotting as described above.

IL-6/sIL-6R COMPLEX FOR PROMOTION OF LIVER FUNCTIONS

FIELD AND BACKGROUND

The present invention relates to a novel method for promoting liver regeneration, and in particular to a method using a composition featuring the IL-6/sIL-6R complex for promoting liver cell proliferation and liver weight restoration, as well as the restoration of liver functions.

The loss of liver functions from traumatic or toxic injury or disease may cause severe debilitation or even death. There are many causes for the loss of liver functions, including malignancies in the liver, both primary and those which metastasize to the liver from another location in the body, viral diseases such as the many forms of viral Hepatitis, and hepatotoxicity caused by exposure to excessive liver toxins such as drug overdose and pesticides. Indeed, even some normally non-toxic substances can become hepatotoxic when abused, such as paracetamol and ethanol. Thus, injury to the liver, resulting in the loss of liver functions, can have many different initial causes.

Patients with acute liver failure have high morbidity and mortality rates. Only 40% of patients treated with conservative medical treatment alone survive. Those patients which do survive are somehow able to restore liver functions. Among the essential functions of the liver are glucose regulation, synthesis of many blood proteins like albumin and coagulation proteins, secretion of bile, biodegradation of toxic compounds, and others[1] (see Appendix for a complete list of references). Little if any disturbance is observed in these functions when only 33% of the liver remains intact and 90% of the remaining cells undergo proliferation and regeneration[2].

Liver regeneration is important for the restoration of liver functions in response to injury, either disease or trauma induced. The term "liver regeneration" is defined as an orchestrated response induced by specific external stimuli and involving sequential changes in gene expression, growth factor production, and morphological structure[3]. Studies have shown that when rats are joined in pairs through parabiotic circulation, hepatectomy of one member of the pair causes regeneration of the intact liver of the other member, with the maximum effect seen when the liver of one animal is totally removed[2,3]. As demonstrated by these and other studies, many soluble factors, such as multiple growth factors and cytokines, are mitogenic signals for hepatocytes during liver regeneration.

Several lines of evidence suggest that TNF-α (Tumor necrosis factor alpha) and IL-6 (Interleukin-6) are the most crucial components of the early signaling pathways leading to regeneration. IL-6 is secreted by Kupffer cells, and this secretion is stimulated by TNF-α. IL-6 is an important signal for the initiation of acute phase protein synthesis by hepatocytes as a part of the overall inflammatory response[4]. Recent experiments have demonstrated that liver regeneration following partial hepatectomy (pHx) is massively impaired in mice carrying a homozygous deletion of the IL-6 gene[5] or of the TNF-α type I receptor gene[6]. Furthermore, the plasma IL-6 concentration increases after pHx (partial hepatectomy), reaching high levels by 24 hours after the removal of liver tissue[2,7]. Thus, IL-6 and TNF-α are important components of the response to liver injury.

On target cells, IL-6 first binds to a specific IL-6 receptor[12]. This IL-6/sIL-6R complex induces the homodimerization of two gp130 signal transducing molecules[13, 14] leading to intracellular signaling events. Soluble forms of the IL-6R (sIL-6R) are generated by limited proteolysis from the cell surface[15] and render cells which do not express membrane bound IL-6R responsive towards IL-6[16]. Furthermore, sIL-6R acts as a serum-binding protein for IL-6 and prolongs the plasma half-life of IL-6[17]. The presence of the IL-6/sIL-6R complex in IL-6/sIL-6R double transgenic mice leads to a marked extramedullary expansion of hematopoietic progenitor cells[18]. The presence of IL-6 alone does not cause similar effects. Thus, the IL-6/sIL-6R appears to have certain effects which extend beyond those of IL-6 alone.

Unfortunately, in spite of many published findings regarding the effects of IL-6 and other molecular components of the liver regeneration pathway, few suitable treatments are available for those suffering from a loss of liver functions. Liver transplantation is the only established therapy which has been shown to improve the survival rate of patients with acute liver failure. However, transplantation is a time consuming and costly therapy associated with a life-long requirement for immunosuppression. Long-term side-effects of the immunosuppression remain unevaluated. In addition, as for any type of organ transplantation, suitable donors are not always available.

Therapies based on the molecular basis of liver regeneration have been examined in an attempt to the development of new treatment strategies beyond liver transplantation. For example, experiments in which recombinant HGF (hepatocyte growth factor) was administered to animals following liver injury suggested that the administration of HGF might be beneficial in liver regeneration and that HGF might help to improve the regenerating capacity of the liver[20-22]. However, since the plasma half-life of HGF is extremely short (1 1/2 of 4.5 min), HGF must be administered by a continuous infusion into a peripheral vein or into the portal vein. Such inconveniently frequent administration is a severe drawback to HGF treatment.

There is therefore a need for, and it would be useful to have, a novel treatment for liver injury which would promote the restoration of liver functions in a subject by promoting and enhancing liver regeneration.

SUMMARY OF THE INVENTION

It is one object of the present invention to provide a treatment for the promotion of liver regeneration and the restoration of liver functions in a subject suffering from liver injury.

It is another object of the present invention to provide such a treatment which is effective on the molecular level, through the stimulation of the endogenous liver regeneration pathways and mechanisms of the subject.

It is still another object of the present invention to provide such a treatment through the provision of a pharmaceutically effective composition which features the IL-6/sIL-6R complex.

It is yet another object of the present invention to provide such a composition which includes Hyper-IL-6.

These and other objects of the present invention are explained in greater detail in the description, Figures and claims below.

The methods of treatment of the present invention involve the administration of a composition which features the IL-6/sIL-6R complex, especially Hyper-IL-6. As described in further detail below, this complex is able to promote liver regeneration and the restoration of liver functions, and is able to significantly increase longevity when administered

exogenously to subjects suffering from liver injury. The background art has neither taught nor suggested any effect for the complex in subjects which do not express the components of the complex endogenously. Furthermore, the background art has certainly neither taught nor suggested any effect for the complex when added exogenously to subjects following liver injury. Thus, the effects of the composition and methods of the present invention are unexpected and are not taught or suggested by the background art.

According to the teachings of the present invention, there is provided a method for treating an injury to a liver of a subject, comprising the step of administering, to the subject, a pharmaceutically acceptable amount of an IL-6/sIL-6R complex in a pharmaceutically acceptable carrier, such that the injury to the liver is treated.

Preferably, the IL-6/sIL-6R complex includes Hyper-IL-6. Also preferably, the IL-6/sIL-6R complex is administered to the subject parenterally.

According to preferred embodiments of the present invention, the injury to the liver is selected from the group consisting of damage caused by a toxic substance, damage caused by mechanical trauma, damage caused by a malignancy, damage caused by an autoimmune pathological process, and damage caused by a pathogen. Preferably, the damage caused by the toxic substance includes alcoholic hepatitis and drug induced hepatopathology. Also preferably, the pathogen is a Hepatitis virus. Also preferably, the injury to the liver is selected from the group consisting of acute liver failure and chronic liver failure.

According to another embodiment of the present invention, there is provided a composition for treating an injury to a liver, comprising a pharmaceutically effective amount of an IL-6/sIL-6R complex in a pharmaceutically acceptable carrier, the pharmaceutically effective amount being an amount sufficient for treating the injury to the liver.

Preferably, the IL-6/sIL-6R complex is Hyper-IL-6.

Hereinafter, the term "injury to the liver" includes but is not limited to liver damage caused by toxic substances, by mechanical disruption or trauma, by a malignancy whether primary or metastasizing from another body tissue, by an autoimmune or other genetically-related pathological process, or by a pathogen such as any of the group of Hepatitis viruses. The term "injury to the liver" also encompasses acute or chronic liver failure, as well as conditions in which liver failure has not occured.

Hereinafter, the term "IL-6/sIL-6R complex" refers both to a bimolecular protein complex which features both the IL-6 polypeptide and sIL-6R, the soluble IL-6 receptor protein, and to a unimolecular protein which includes the bioactive portions of IL-6 and sIL-6R connected with a flexible linker, substantially as previously described in PCT Patent Application No. PCT/DE97/00458 and in Fischer, M. et al., *Nature Biotech.* 15, 142–145 (1997), incorporated by reference as if fully set forth herein, as well as any biologically active equivalents thereof.

Hereinafter, the term "Hyper-IL-6" refers to a unimolecular protein which includes the bioactive portions of IL-6 and sIL-6R connected with a flexible linker, substantially as previously described and shown in FIG. 1 of PCT Patent Application No. PCT/DE97/00458 (referred to as "H-IL-6" in that reference).

Hereinafter, the term "biologically active" refers to molecules, or complexes thereof, which are capable of exerting an effect in a biological system.

Hereinafter, the term "amino acid" refers to both natural and synthetic molecules which are capable of forming a peptide bond with another such molecule. Hereinafter, the term "natural amino acid" refers to all naturally occurring amino acids, including both regular and non-regular natural amino acids. Hereinafter, the term "regular natural amino acid" refers to those amino acids which are normally used as components of a protein. Hereinafter, the term "non-regular natural amino acid" refers to naturally occurring amino acids, produced by mammalian or non-mammalian eukaryotes, or by prokaryotes, which are not usually used as a component of a protein by eukaryotes or prokaryotes. Hereinafter, the term "synthetic amino acid" refers to all molecules which are artificially produced and which do not occur naturally in eukaryotes or prokaryotes, but which fulfill the required characteristics of an amino acid as defined above. Hereinafter, the term "peptide" includes both a chain of a sequence of amino acids of substantially any of the above-referenced types of amino acids, and analogues and mimetics having substantially similar or identical functionality thereof.

With regard to the unimolecular protein, such as Hyper-IL-6, and the bimolecular protein complex, the expression "linker" relates to linkers of any kind, which are suitable for the binding of polypeptides. Examples of such linkers include but are not limited to bifunctional, chemical cross-linkers; a disulfide-bridge connecting two amino acids of both polypeptides; and a peptide of polypeptides.

The bimolecular protein complex includes both IL-6 and sIL-6R, as well as biologically active portions and variants thereof, connected by a linker. The term "variants" includes any homologous peptide to either IL-6 or sIL-6R, for example including any amino acid substitution or substitutions which still maintain the biological activity of the original peptide or a polypeptide which directly stimulates the membrane receptor for the IL-6/sIL-6R complex which is called gp130.

The unimolecular protein can be a fusion polypeptide. For example, polypeptides featuring the bioactive portions of IL-6 and sIL-6R can be fused with each other and the linker can be a disulfide-bridge produced by the two polypeptides. Preferably the linker is a polypeptide, which connects the two other polypeptides with each other. These fusion polypeptides include a human sIL-6R-polypeptide, which is the extracellular subunit of an interleukin-6-receptor and a human IL-6-polypeptide, whereby the polypeptides are connected by different polypeptide-linkers with each other. The accession number for IL-6 is M14584 (GenBank Protein Sequences Database), and for the soluble IL-6 receptors is M57230 and M20566.

A variation of the unimolecular protein, which includes only amino acids 114–323 inclusive from the sIL-6R-polypeptide, is also included. A second variation includes amino acids 113–323 inclusive of the sIL-6R-polypeptide and amino acids 29–212 of the IL-6-polypeptide. Other variations and combinations as previously disclosed in PCT Patent Application No. PCT/DE97/00458 and in Fischer, M. et al., *Nature Biotech.* 15, 142–145 (1997) are also included in the unimolecular protein embodiment of the IL-6/sIL-6R complex.

Hereinafter, the term "treatment" includes both the amelioration or elimination of an existing condition and the prevention of the genesis of a condition. Hereinafter, the term "Hepatitis virus" includes any virus known to cause viral hepatitis including, but not limited to, Hepatitis A, B, C, D, E and other variants thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 shows that Hyper-IL-6 causes an accelerated reconstitution of liver weight following partial hepatectomy in mice; immediately following a 50% partial hepatectomy, IL-6 (20 μg/mouse) or Hyper-IL6 (2 μg /mouse), or physiological saline was injected intraperitoneally into mice. At the time points indicated in the figure, mice were sacrificed, the remnant livers were removed and the percentage of liver weight increase compared to time 0 at hepatectomy was determined (see Methods). Four to six mice were operated at each time point in each treatment group. Mean values ± standard deviations are presented.

FIG. 2 shows that Hyper-IL-6 significantly accelerates liver proliferation in mice following partial hepatectomy; Following a 50% partial hepatectomy, IL-6 (20 μg/mouse) or Hyper-IL6 (2 μg /mouse), or physiological saline was injected intraperitoneally into mice. One hour before the mice were sacrificed, 50 mg/kg body weight BrdU in PBS was injected intraperitoneally into the mice. After removal of the remnant livers, the organs were fixed in 4% formaldehyde and embedded in paraffin. Tissue sections were subjected to BrdU immunohistochemistry. The percentage of BrdU-positive nuclei were counted in at least three mice per treatment group. Mean values ± standard deviation are shown.

FIG. 3 shows BrdU labelling following partial hepatectomy in mice; Immunohistochemical detection of BrdU incorporation in S-phase liver nuclei as an indicator of liver cell proliferation. Following 50% partial hepatectomy, mice were either left untreated (A-C), treated with 20 μg IL-6/mouse (A'-C'), or treated with 2 μg Hyper-IL-6 (A"-C"). Mice were sacrificed 24 hours (A, A', A"), 36 hours (B, B', B"), or 120 hours (C, C', C") following surgery. One hour before the animals were sacrificed 50 mg/kg body weight BrdU in PBS was injected intraperitoneally. The bars represent 100 μm. and

FIG. 4 shows that the acute phase response is intact in mice following partial hepatectomy. Upper panel: 24 hours after intraperitoneal injection of saline, 20 μg IL-6 alone, or 2 μg Hyper-IL-6, blood was drawn from the animals which did not undergo partial hepatectomy. One μl of the murine serum was loaded on a 12.5% SDS gel and was subjected to SDS-PAGE. The gel was blotted onto a nitrocellulose membrane and was subjected to Western blotting using a monoclonal antibody specific for murine haptoglobin. Lower panel: Mice that had undergone partial hepatectomy were immediately treated with either saline, 20 μg IL-6, or with 2 μg Hyper-IL-6.24 hours after the operation, blood was drawn from the animals and serum was subjected to Western blotting as described above.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

The present invention is drawn towards novel methods of use of the IL-6/sIL-6R complex, such as Hyper-IL-6. The IL-6/sIL-6R complex has now been shown to promote liver regeneration and the restoration of liver functions in subjects suffering from liver injury (see Example 1). Furthermore, administration of the IL-6/sIL-6R complex has now been shown to increase the lifespan of these subjects, as compared to untreated subjects (see Example 2). Thus, the IL-6/sIL-6R complex has significant utility for the treatment of many different types of liver diseases.

The principles and operation of the methods of treatment which feature IL-6/sIL-6R complex according to the present invention may be better understood with reference to the non-limiting illustrative examples below.

EXAMPLE 1

Effects of the IL-6/sIL-6R Complex in a Mouse Experimental Model

The effects of Hyper-IL-6 were examined in a mouse experimental model of liver damage. Two groups of mice underwent partial hepatectomy, in which a portion of the liver was removed, thereby seriously reducing overall liver functions. One group of mice received Hyper-IL-6, while a second, control group did not. The mice treated with Hyper-IL-6 showed and increased rate of liver regeneration and of a substantial restoration of liver functions. By contrast, the control mice showed a much slower rate of restoration of liver functions through liver regeneration. The experimental methods were as follows.

First, recombinant human IL-6[39] and Hyper-IL-6[19] were prepared as described in these references (39 and 19, respectively).

For partial hepatectomy, C57xB1/6 mice of 8 weeks old were obtained from the animal facility of the University of Mainz, Germany. In an initial experiment the relation between total body weight and liver weight was established in 20 mice. The mean total body weight was 27.45±1.25 g. The mean liver weight was 1.2±0.056 g. The mean ratio of liver weight (LW)/mean total body weight (BW) was 0.045.

Partial hepatectomy was performed as described by Higgins and Anderson[40]. Briefly, at the day of the operation, food was withdrawn at 8 AM in the morning and the surgery was carried out between 6 and 8 PM. Animals were anesthetized by an intraperitoneal injection of 2.5% avertin (mixture of 10 grams of tribromoethyl alcohol and 10 ml tertiary amyl alcohol). The total body weight of each mouse was recorded. The mice were then subjected to midventral laparotomy with an approximately 50% liver resection (left lateral and left half of medial lobes), slightly modified according to the procedure originally described by Higgins and Anderson[40]. The weight of the removed liver lobes was recorded. The weight of the residual liver lobes left behind was calculated by application of the formula 0.045=LW/BW. This weight was designated as "liver weight 1 at time 0". After the operation, the peritoneum was sutured and the skin was closed with wound clips.

Immediately after surgery, three groups, each including four to six mice, was subjected to one of three treatments: 2 μg Hyper-IL-6, 30 μg IL-6, or no treatment (control). All treatments were administered by injection; the control mice received an injection of physiological saline. After different time points as indicated in the figures, the mice were killed by cervical dislocation. The residual enlarged lobes were totally removed and their weight was recorded. This weight was designated as "liver weight 2 at time x". The percentage of the change, increase or decrease, in the weight of the liver after a defined period of time was calculated by subtracting liver weight 1 from liver weight 2.

In order to label the tissues with BrdU (5-bromo-2'-deoxyuridine), the animals were injected intraperitoneally with BrdU (50 mg/kg) (0.2% solution in PBS) one hour before the remnant liver was harvested and fixed. BrdU is a thymidine-analogue which is incorporated during the S-phase of the cell cycle into DNA. Applying immunhistochemical analysis using anti-BrdU-antibodies, S-phase-nuclei can be specifically detected. BrdU had been previously shown to be incorporated in S-phase-nuclei to the same extent as [3H]-Thymidine[20]. One hour after injection, the liver was then removed and immediately fixed in 4% paraformaldehyde (pH 7.2) at 4° C. An automated tissue

processor was used to embed the livers in paraffin. Tissue sections (5 microns) were cut on a microtome and adhered to poly-lysine-coated glass slides. Staining of fixed tissue samples was carried out using an antibody to BrdU (Boehringer Mannheim) enabling proliferating cells (red nuclei) to be distinguished from quiescent ones (blue nuclei). The immunhistochemical study was performed as suggested by the manufacturer (BrdU labelling and detection kit by Boehringer Mannheim) and as described previously[31].

For the determination of haptoglobin protein determination in the serum, one microliter of murine serum was loaded on a 12.5 SDS gel and was subjected to SDS-PAGE gel analysis. The gel was blotted onto a nitrocellulose membrane and was subjected to Western blot analysis using a rabbit anti-human haptoglobin antibody (Dako, Glostrup, Denmark).

Results

The results demonstrated that Hyper-IL-6 causes an accelerated reconstitution of the liver weight following partial hepatectomy when compared to control mice and mice treated with IL-6, as shown in FIG. 1. Both untreated and IL-6-treated mice had a comparable increase of their liver weight. At 36 and 72 hours post surgery, IL-6-treated mice had slightly higher liver weights compared to untreated mice, which was not statistically significant. In Hyper-IL-6-treated mice, however, there was a dramatic increase of the liver weight at 72 and 120 hours post surgery ($p<0.005$). At 168 hours, the liver weights of all treatment groups had reached their baseline weight. These data demonstrate for the first time that the presence of the IL-6/sIL-6R-complex is able to significantly increase the liver weight restoration following partial removal of the liver. Most remarkably, IL-6 alone did not improve the rate of liver weight increase in mice in this experimental model.

FIG. 2 demonstrates that the increased rate of weight gain found in mice receiving Hyper-IL-6 treatment is caused by the significant acceleration of liver proliferation in these mice, when compared to control mice and mice receiving IL-6 treatment. Control and IL-6-treated animals showed a peak of BrdU-labelled cells at 72 and 120 hours post surgery. By contrast, in Hyper-IL-6 treated mice, the maximal percentage of BrdU-positive cells was detected as early as 24 and 36 hours post surgery. The difference was highly statistically significant. The results demonstrate that the presence of the IL-6/sIL-6R-complex markedly accelerates liver proliferation which results in the fast restoration of the liver weight.

FIG. 3 shows representative immunohistochemical studies after 50% partial hepatectomy (untreated (A-C), treated with 20 μg IL-6/mouse (A'-C'), or treated with 2 μg Hyper-IL-6 (A"-C"); sacrificed 24 hours (A, A', A"), 36 hours (B, B', B"), or 120 hours (C, C', C") following surgery). The bars represent 100 μm. As shown, control mice and IL-6-treated mice do not have any BrdU-positive cells detectable at 24 and 36 hours post surgery. However, at these time points, in Hyper-IL-6-treated mice, there is a high number of BrdU-labelled cells.

FIG. 4 shows that the hepatic acute phase protein production is intact after partial hepatectomy, as determined by analysis of the serum haptoglobin concentration by Western blot analysis in blood samples. First, the serum haptoglobin concentrations were measured in serum samples from mice which did not undergo partial hepatectomy. These mice were injected with saline, IL-6 alone (20 μg), or Hyper-IL-6 (2 μg). The upper panel of FIG. 4 shows that 24 hours after injection, IL-6 treatment alone leads to some slight haptoglobin protein increase in the serum, whereas the treatment with Hyper-IL-6 resulted in a marked elevation of the haptoglobin concentration in the serum. The haptoglobin mRNA concentration in the liver corresponds with the protein data shown in FIG. 4 (data not shown).

When the haptoglobin serum concentration was determined in hepatectomized mice 24 hours following the operation, mice receiving saline injections also had a slight haptoglobin protein elevation in their serum when compared to mice which did not undergo hepatectomy. The treatment with IL-6 alone and with Hyper-IL-6 resulted in a comparable serum haptoglobin concentration when compared to mice which did not undergo hepatectomy (FIG. 4, lower panel). These data show that the regenerating liver is capable of mounting a normal acute phase protein response.

These data have demonstrated for the first time that in the presence of IL-6 and its soluble receptor, sIL-6R, liver regeneration in mice following partial hepatectomy is greatly accelerated. Moreover, the IL-6/sIL-6R complex has now been shown to rapidly cause hepatocyte proliferation of the liver following partial hepatectomy in mice. IL-6 alone at a ten-fold higher dosage than the designer cytokine Hyper-IL-6 did not cause accelerated liver regeneration or hepatocyte proliferation as compared to untreated animals. Thus, only Hyper-IL-6 was able to induce liver regeneration and the restoration of liver functions in mice which had undergone partial hepatectomy.

EXAMPLE 2

Survival of Rats with Hepatatic Failure

In order to assess the ability of Hyper-IL-6 to treat fulminant hepatatic failure (FHF), FHF was induced in four rats. Two also received Hyper-IL-6, one received hepatocytes and one received human IL-6 alone. The rats which received Hyper-IL-6 survived for over one month, while the other rats died within 24–72 hours. The experimental method was as follows.

Four male Sprague-Dawley rats were deprived of food, but not of drinking water, for 12 hours. Next, the rats were injected i.p. with D-Galactosamine (1.4 g/kg, pH=6.8). After 24 hours of D-Galactosamine treatment, fulminant hepatic failure was induced. Two rats then received Hyper-IL-6 (7 micrograms, i.p.). One rat received human IL-6 (80 micrograms, i.p.). One rat received a transplantation of syngeneic hepatocytes (2×10^8 cells).

The rat which received human IL-6 died 24 hours after treatment, while the rat which received the hepatocyte transplant died within 72 hours. Only the animals treated with Hyper-IL-6 survived for over one month before being sacrificed. Thus, Hyper-IL-6 was clearly able to prolong the life-span of rats suffering from hepatic failure.

EXAMPLE 3

Compositions and Methods of Treatment with Hyper-IL-6

As described previously in the section entitled "Summary of the Invention", the term "IL-6/sIL-6R complex" refers to a bimolecular protein complex which features both the IL-6 polypeptide and sIL-6R, the soluble IL-6 receptor protein; and to a unimolecular protein which includes the bioactive portions of IL-6 and sIL-6R connected with a flexible linker, as previously described in PCT Patent Application No. PCT/DE97/00458, and in Fischer, M. et al., *Nature Biotech.* 15, 142–145 (1997), as well as pharmaceutically acceptable salts thereof.

The composition containing the IL-6/sIL-6R complex, and in particular Hyper-IL-6, can be administered to a subject in a number of ways, which are well known to the art. Hereinafter, the term "subject" refers to the human or lower animal to whom Hyperkine was administered. For example, administration may be done topically (including ophthalmically, vaginally, rectally, intranasally and by inhalation), orally, or parenterally, for example by intravenous drip or intraperitoneal, subcutaneous, or intramuscular injection.

Particularly preferred routes of administration include parenteral, intranasal and by inhalation.

Formulations for topical administration may include but are not limited to lotions, ointments, gels, creams, suppositories, drops, liquids, sprays and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, sachets, capsules or tablets. Thickeners, diluents, flavorings, dispersing aids, emulsifiers or binders may be desirable.

Formulations for parenteral administration may include but are not limited to sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

Dosing is dependent on the severity of the symptoms and on the responsiveness of the subject to the IL-6/sIL-6R complex, as well as on the particular embodiment administered. Persons of ordinary skill in the art can easily determine optimum dosages, dosing methodologies and repetition rates.

As noted previously, the compositions found to be useful in the methods of the present invention include the IL-6/sIL-6R complex. The methods of the present invention are useful for the treatment of injury to the liver. The following example is an illustration only of a method of treating such an injury to the liver, and is not intended to be limiting in any way.

The method includes the step of administering the composition including the IL-6/sIL-6R complex, in a pharmaceutically acceptable carrier as described above, to a subject to be treated. The composition preferably features Hyper-IL-6 as the embodiment of the IL-6/sIL-6R complex. The composition is administered according to an effective dosing methodology, preferably until a predefined endpoint is reached, which could include one or more of the following: a normalized level of coagulation factors 5 or 7; normalized prothrombin time; the absence of hepatic encephalopathy; normalized levels of liver enzymes such as aspartate aminotransferase and alanine aminotransferase; and normalized ammonia levels.

In a preferred embodiment of the method of the present invention, the composition including the IL-6/sIL-6R complex is administered to a subject before, during or after liver transplantation, or a combination of these timepoints of administration thereof, in order to promote growth and regeneration of the transplanted liver.

Examples of injuries to the liver for which such a method of treatment would be suitable include but are not limited to liver damage caused by toxic substances, including alcoholic hepatitis and drug induced hepatopathy; damage caused by mechanical disruption of trauma; damage caused by a malignancy, whether primary or metastasizing from another body tissue; damage caused by an autoimmune or other genetically-related pathological process; and damage caused by a pathogen such as any of the group of Hepatitis viruses, including dominant viral hepatitis. The term "injury to the liver" also encompasses acute or chronic liver failure, including fulminant hepatic failure, as well as conditions in which liver failure has not occured, including any condition featuring a reduction of liver functions from a substantially normal level.

The term "treating" includes ameliorating, alleviating or substantially eliminating a liver injury, as well as substantially preventing a liver injury.

While the invention has been described with respect to a limited number of embodiments, it will be appreciated that many variations, modifications and other applications of the invention may be made.

What is claimed is:

1. A method for treating an injury to a liver of a subject, comprising the step of administering, to the subject, a pharmaceutically acceptable amount of an IL-6/sIL-6R complex in a pharmaceutically acceptable carrier, such that the injury to the liver is treated.

2. The method of claim 1, wherein said IL-6/sIL-6R complex is Hyper-IL-6.

3. The method of claim 1, wherein said IL-6/sIL-6R complex is administered to the subject parenterally.

4. The method of claim 1, wherein the injury to the liver is selected from the group consisting of reduction of liver function from a normal level caused by a toxic substance, reduction of liver function from a normal level caused by mechanical trauma, reduction of liver function from a normal level caused by a malignancy, and reduction of liver function from a normal level caused by a pathogen.

5. The method of claim 4, wherein said reduction of liver function from a normal level caused by said toxic substance includes alcoholic hepatitis and drug induced hepatopathy.

6. The method of claim 4, wherein said pathogen is a Hepatitis virus.

7. The method of claim 4, wherein the injury to the liver is selected from the group consisting of acute liver failure and chronic liver failure.

* * * * *

Liver regeneration induced by a designer human IL-6/sIL-6R fusion protein reverses severe hepatocellular injury

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ABSTRACT **The cytokine IL-6 plays a significant role in liver regeneration in conjunction with additional growth factors (HGF, TNF-α, and TGF-α). Many IL-6 effects depend on a naturally occurring soluble IL-6 receptor (sIL-6R). Here, the chimeric protein hyper-IL-6, constructed from the human IL-6 protein fused to a truncated form of its receptor, was found to have superagonistic IL-6 properties, and as such, enhanced liver cell regeneration. Hyper-IL-6 reversed the state of hepatotoxicity and enhanced the survival rates of rats suffering from fulminant hepatic failure after D-galactosamine administration. The hyper-IL-6 protein has a significant potential for use in the treatment of severe human liver diseases.—Galun, E., Zeira, E., Pappo, O., Peters, M., Rose-John, S. Liver regeneration induced by a designer human IL-6/sIL-6R fusion protein reverses severe hepatocellular injury. *FASEB J.* 14, 1979–1987 (2000)**

Key Words: interleukin 6 · chimeric protein · hyper-IL-6 · cytokines · hepatotoxicity · liver failure

FULMINANT HEPATIC FAILURE (FHF) is a clinical condition with a high mortality rate: more than half of the patients suffering from the devastating clinical condition will not survive without emergency liver transplantation (LTx) (1). Very few of those patients in need of LTx will be able to undergo this therapy in due time, and even those who do have an expected 1 year survival rate after transplantation of only ~60% (1). Various preventive measures have been suggested for FHF (2–5), but these are of little use since severe hepatotoxicity or FHF are unpredicted events and therefore can only be treated when diagnosed.

One of the causes of death by FHF is liver cell apoptosis or hepatocyte death through necrosis, as seen in the case of acute hepatitis A virus infection (6). Thus, FHF might be cured by factors that could induce liver cell regeneration (7–10). It has recently been reported that liver cell proliferation is also enhanced by interleukin 6 (IL-6) (11, 12). IL-6 binds to cells through the IL-6 receptor α (IL-6Rα, gp80), thus facilitating its interaction with a second IL-6 receptor molecule, IL-6Rβ (gp130). IL-6Rα can also be found as a soluble protein that is thought to be generated either by limited proteolysis of the membrane-bound receptor (13) or by translation of an alternatively spliced mRNA (14). In a process called 'trans-signaling', soluble IL-6R (sIL-6R) has been shown to sensitize target cells (15) and to cause cells that do not express membrane-bound IL-6Rα to be responsive to IL-6 (16, 17). Marked hepatocellular hyperplasia is seen in IL-6/sIL-6 receptor (IL-6/sIL-6R) double transgenic mice, but not in single transgenic IL-6 mice, suggesting that sIL-6R recruits IL-6-unresponsive hepatocytes to proliferation (18, 19). Recently, a fusion protein called hyper-IL-6 was constructed, consisting of human IL-6 and the human sIL-6R connected by a flexible peptide chain (20). Hyper-IL-6 was shown to exhibit a high activity level on gp130-expressing cells both *in vitro* (20) and *in vivo* (21). It appears that the hyper-IL-6 fusion protein acts as a superagonist by simulating the interaction between IL-6 and sIL-6Rα (20).

In the present study, we have assessed the effects of hyper-IL-6 in inducing hepatocellular proliferation and liver cell regeneration *in vivo*, with the aim of determining its potential therapeutic value.

MATERIALS AND METHODS

Construction, synthesis, and purification of the recombinant fusion protein hyper-IL-6

We used a cassette consisting of human sIL-6R cDNA (corresponding to amino acid residues 115–323) and human IL-6 cDNA (corresponding to amino acid residues 29–212) fused by a synthetic DNA linker coding for the amino acid sequence Arg-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Val-Glu. We con-

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structed this cassette using polymerase chain reaction technology; using the restriction enzymes *Sma*BI and *Eco*RI, we then cloned it into the *Pichia pastoris* expression vector pPIC9 (Invitrogen, San Diego, Calif.). Cleavage of the signal peptide after secretion by transfected yeast cells leads to the secretion of the fusion protein hyper-IL-6 with an NH_2-terminal extension of 8 amino acid residues (Glu-Lys-Arg-Glu-Ala-Glu-Ala-Tyr). The recombinant hyper-IL-6 (H-IL-6) protein was purified from yeast supernatants by anion-exchange chromatography and gel filtration and then visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. The circular dichroism spectra of H-IL-6 (solvent: PBS; pH 7.4) revealed the expected secondary structure content of the fusion protein (20).

D-gal rat nonlethal severe hepatotoxicity model

We slightly modified (see below) the male Fischer rat model in which a state of nonlethal severe hepatotoxicity was created by the application of D-galactosamine (D-gal). To determine the level of hepatotoxicity, we used the following clinical biochemical criteria: *1)* blood glucose levels, since hypoglycemia is induced by the failure of liver homeostatic mechanisms; *2)* bilirubin levels, since jaundice often accompanies acute liver injury; *3)* the blood level of alanine transferase (ALT), since such hepatocyte cytosolic enzyme transaminases are released into the blood when liver cells are injured. In addition, since most blood coagulation factors are produced in the liver, in severe acute liver disease the production of coagulation factors is typically reduced. Coagulation factors V and VII have a short $t_{1/2}$ of less than 8 h, so we chose to measure their serum levels to follow the course of the liver injury. Before we administered D-gal, the results of the liver function tests were as follows (mean ± SD, $n=8$): glucose 84±19 mg%; coagulation factor V 119± 41%; coagulation factor VII 98±10%; ALT 55±4 I.U., and bilirubin 4± 0 μM/l.

For these studies we used male Fisher rats weighing 150–200 g (Harlan, Animal Breeding Center, Jerusalem, Israel) that were fed a standard diet of rat chow and tap water and housed in standard facilities at room temperature of 25°C with a 12 h day/night cycle. After the rats had fasted for 12 h, acute liver damage was induced by the intraperitoneal (i.p.) administration of 100–500 mg/kg body weight doses of D-gal (Sigma Chemical Co., G0264, Israel) dissolved in 0.9% NaCl and adjusted with 1N NaOH to pH 6.8. After the injection of D-gal, the rats were fasted for an additional 12 h, but were provided with water containing 10% glucose *ad libitum* to maintain the blood glucose level. To determine the optimal dose of D-gal for inducing severe but nonlethal hepatotoxicity, five groups of two rats each received a single i.p. dose of 100 mg, 200 mg, 300 mg, 400 mg, or 500 mg/kg body weight injection of D-gal. The results of a D-gal dose response experiment (Fig. 1) revealed that these rats developed the hepatotoxic effect after the i.p. administration of 300 mg/kg of D-gal. We used this dosage in later experiments designed to assess the effects of hyper-IL-6, IL-6, and glucose treatments (Fig. 2); see below.

D-gal male Fischer rat FHF model

The dosage of D-gal required for 100% mortality of male rats was revealed in a dose response experiment in which male Fischer rats were injected i.p. with D-gal at dosages of 1, 1.2, or 1.4 gm/kg body weight (Fig. 3). In similar experiments on female Fischer rats (animal weight 150–170 g), the dose response survival experiment was conducted with doses range between 100 and 1400 mg/kg. All the female animals died when treated with D-gal at a dose of 800 mg/kg or higher.

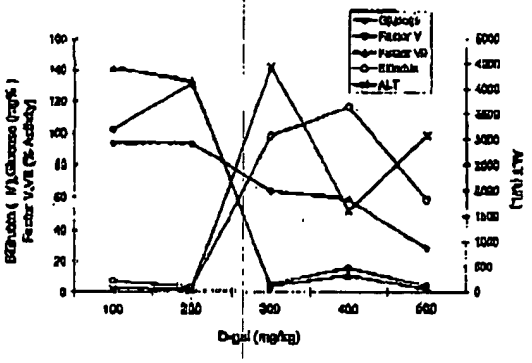

Figure 1. The optimal dosage of D-gal for inducing nonlethal acute liver damage was 300 mg/kg. To determine the optimal dose of D-gal for inducing severe hepatotoxicity, a single i.p. dose of D-gal at a dosage of 100 mg, 200 mg, 300 mg, 400 mg, or 500 mg/kg body weight was injected into each member of five pairs of rats. After D-gal administration, the rats were fasted except for 10% glucose in water *ad libitum* to permit the maintenance of blood glucose levels. After 12 h, the level of hepatotoxicity was evaluated as reflected by hypoglycemia (◆), decreases in blood coagulation factors V (●) and VI (▲), increased bilirubin as an indication of jaundice (○), and increased blood levels of alanine transferase (ALT) (x).

Assessment of liver cell proliferation by 5-bromo-2'-deoxyuridine (BrdU) incorporation

We assessed liver regeneration as reflected by cell proliferation measured by BrdU immunohistological staining (see Fig. 5). One and 2 h before killing animals, an i.p. injection of a PBS solution of the thymidine analog 5-bromo-2'-deoxyuridine (Sigma Chemical Co.) was administered at a dose of 50 mg/kg body weight. Livers were harvested and fixed in 4% formaldehyde buffer. An automated tissue processor was used for fixation, followed by liver embedding in paraffin. Tissue sections (4–6 μ) were cut on a microtome and adhered to poly-L-lysine-coated glass slides. Staining of fixed tissue samples was carried out using an antibody to BrdU (Zymed, San Francisco, Calif.), enabling the proliferating cells (red nuclei) to be distinguished from others (blue nuclei). The immunohistochemical study was performed according to the manufacturer's instructions (Zymed BrdU labeling and detection kit).

Thioacetamide (TAA) FHF rat model

A TAA dose response study was conducted with male Sprague-Dawley rats (weight 180–220 g) that received TAA in 5 ml saline administered i.p. daily for 3 consecutive days. To prevent hypoglycemia and hypokalemia, at 12 h intervals after the initial TAA injection the animals were treated with subcutaneous injections of 25 ml/kg body weight of 5% dextrose/0.45% saline containing 20 mEq/l KCl; i.p. administration of TAA at 300 mg/kg body weight induced death from FHF in 100% of these male Sprague-Dawley rats.

Assessing liver cell apoptosis with the TUNEL assay

Male Fischer rats were treated with D-gal at a dosage of 300 mg/kg body weight; 7 h later they were treated i.p. with either IL-6 at 80 μg/rat or H-IL-6 at 8 μg/rat. Two days after the D-gal injections, the livers were harvested fixed in 4% form-

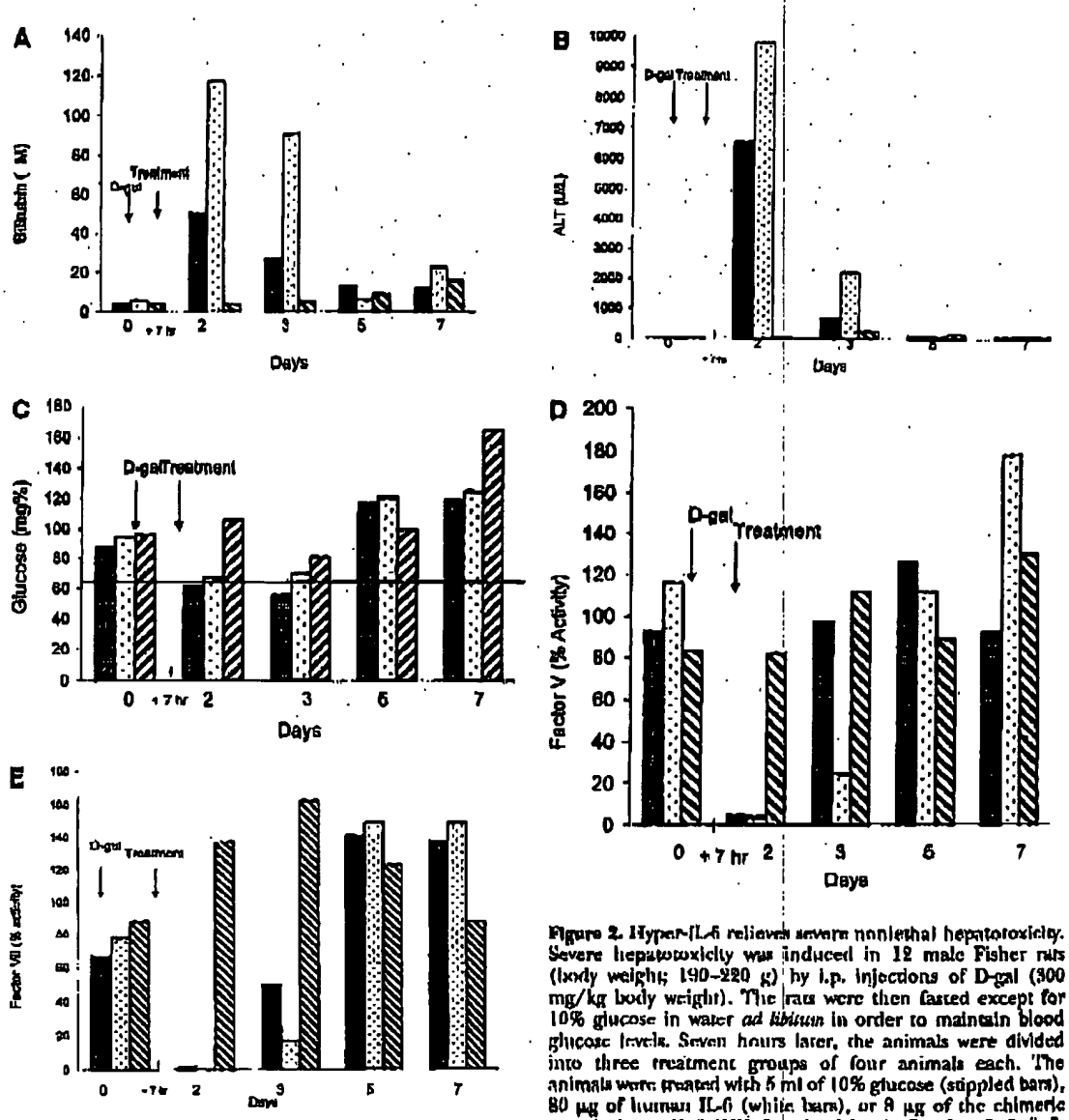

Figure 2. Hyper-IL-6 relieves severe nonlethal hepatotoxicity. Severe hepatotoxicity was induced in 12 male Fisher rats (body weight; 190–220 g) by i.p. injections of D-gal (300 mg/kg body weight). The rats were then fasted except for 10% glucose in water *ad libitum* in order to maintain blood glucose levels. Seven hours later, the animals were divided into three treatment groups of four animals each. The animals were treated with 5 ml of 10% glucose (stippled bars), 80 μg of human IL-6 (white bars), or 8 μg of the chimeric protein hyper-IL-6 (HIL-6; striped bars). On days 0, 2, 3, 5, and 7, an animal from each treatment group was killed and its blood was used to assess liver damage and function as reflected by the blood levels of bilirubin (*A*), ALT (*B*), glucose (*C*), coagulation factor V (*D*), and coagulation factor VII (*E*).

aldehyde and paraffin embedded. Apoptosis was examined in 4 μm sections, as assayed by direct immunoperoxidase to detect digoxigenin-labeled genomic DNA. As a positive control we used sections treated with DNase I to nick all DNA (1 μg/ml, determined after a preliminary dilution experiment); for a negative control, we used sections that were only immersed in a Tdt buffer containing 8 nM of biotin dUTP. The TUNEL staining was prepared according to modified protocol of Boehringer Mannheim, (Indianapolis, Ind.). Visual images of TUNEL-stained slides were captured with a digital camera attached to a microscope. Apoptotic cells were identified at the color threshold set (dark brown) for 3',3-diaminobenzidine tetrahydrochloride.

RESULTS

Establishing a severe hepatotoxicity rat animal model

To assess the effects of the HIL-6 fusion protein on D-gal-induced severe liver damage in rats, we developed a model using Male Fisher rats to which we administered D-gal in a single dose of 300 mg/kg body weight (Fig. 1). We assessed the hepatotoxicity

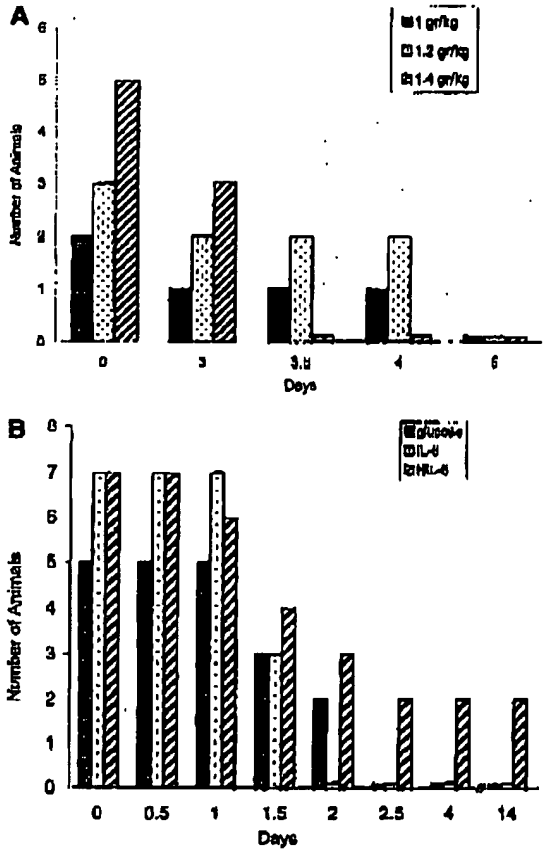

Figure 3. Hyper-IL-6 improves the survival of male rats treated with D-gal to induce lethal FHF. *A)* The survival rate of male Fischer rats (body weight 150–200 g) was monitored twice daily after they were injected i.p. with D-gal at dosages 1 (2 rats), 1.2 (3 rats), or 1.4 (5 rats) g/kg body weight. *B)* Each of 19 male Fischer rats was injected i.p. with D-gal at a fatal dosage of 1.4 g/kg body weight. Seven hours later, the animals were divided into three treatment groups of five, seven, and seven and treated as described for Fig. 2.

of the D-gal by measuring specific liver functions reflected by changes of various molecules in the serum (Fig. 1). D-gal caused the synthetic function of liver cells to be reduced to very low levels as shown by the serum activity of coagulation factors V and VII. The increased levels of serum levels of ALT, a hepatic cytosolic enzyme, and of bilirubin also pointed to liver cell impairment. D-gal-treated rats also suffered from mild hypoglycemia, known to coincide with hepatic failure.

Hyper-IL-6 relieves nonlethal severe hepatotoxicity in a rat model

Using male rats treated with D-gal to induce severe hepatotoxicity, we compared the effects of hyper-IL-6 to those of human IL-6 or of glucose. Seven hours after the i.p. injection of D-gal, we administered hyper-IL-6, IL-6, or glucose (see legend to Fig. 2). The failure of liver homeostatic mechanism is known to be accompanied by various physiological changes, which we monitored in D-gal-treated rats. Jaundice is a common manifestation of acute liver injury, so we measured serum bilirubin levels (Fig. 2*A*); similarly, acute liver damage is accompanied by the release into the blood of hepatocyte cytosolic enzymes the liver transaminases, including ALT (Fig. 2*B*), and of hypoglycemia (Fig. 2*C*). Most blood coagulation factors are produced in the liver, and severe acute liver disease is known to lead to a reduction in the production of coagulation factors (1). Since both the coagulation factors V and VII have a short half-lives ($t1/2 < 8$ h), we chose to follow the synthetic function of the liver by measuring the levels of coagulation factors V (Fig. 2*D*) and VII (Fig. 2*E*). When D-gal-treated rats were subsequently treated with either glucose or IL-6, bilirubin levels remained high (>60 mmol/l) (Fig. 2*A*), as did ALT levels (>6000 IU/l) (Fig. 2*B*) and, to some extent, hypoglycemia (~65 mg%) (Fig. 2*C*), indicating that there was no relief of the severe hepatotoxicity. Furthermore, the production of both coagulation factors V (Fig. 2*D*) and VII (Fig. 2*E*) decreased to below 10% of the normal activity level. Note that from day 5 most of these liver functions returned to normal, suggesting that the effect of the D-gal in this model is transient and is followed by spontaneous liver regeneration. In contrast to the liver functions of the IL-6 and glucose-treated groups, there was no indication of severe hepatotoxicity in the hyper-IL-6-treated group (Fig. 2*A–E*). We observed similar results when we repeated this experiment three times. Thus, it appears that hyper-IL-6 reversed the toxic effect of D-gal. We hypothesized that the reversal of the toxic effect of D-gal by hyper-IL-6 might take place by the induction of liver regeneration.

The effect of hyper-IL-6 on survival of rats with FHF

Having ascertained that HIL-6 reversed the toxic effects of D-gal on liver function (Fig. 2), we wanted to test whether HIL-6 could improve survival after the administration of high lethal doses of D-gal to induce FHF. We injected D-gal at a dosage of 1.4 mg/kg body weight to male Fischer rats and after 7 h administered a single injection of hyper-IL-6, IL-6, or glucose (see legend to Fig. 3). All the animals treated with IL-6 or glucose died after 2.5 days (Fig. 3*B*). Two of the seven animals treated with hyper-IL-6 survived for more than 14 days. We understood these results to suggest that hyper-IL-6 could induce liver regeneration even in a highly stringent FHF rat model.

Hyper-IL-6 but not IL-6 improves survival of female Fischer rats with FHF

To confirm that the results of the experiments with male Fisher rats were not sex biased, we repeated the experiments using female Fischer rats. Seven hours after i.p. D-gal injection at a dosage of 900 mg/kg body weight to induce FHF, these female rats were treated with hyper-IL-6, IL-6, or glucose (dose administered as in previous experiments). After 24 h, all the rats in all the treatment groups were still alive (Fig. 4). However, by day 3 only 1 of the 10 control animals survived (glucose- and IL-6-treated groups). In contrast, 4 of the 5 animals treated with hyper-IL-6 were still alive. These results supported our previous results showing that hyper-IL-6 can prevent rat death caused by liver function failure. We then wanted to explore our hypothesis that hyper-IL-6 acts by stimulating hepatocyte proliferation.

The effect of hyper-IL-6 on hepatocyte proliferation

We examined the livers from female Fischer rats treated first with D-gal and then with hyper-IL-6, IL-6, or glucose as described above (Fig. 4); after 2 or 3 days, the rats were pulsed with BrdU for immunohistological staining (Fig. 5). We made more than 10 low-power field microscopic examinations. One day after D-gal injection, 40–50% of the hepatocytes in the hyper-IL-6-treated group were BrdU positive (Fig. 5*C, F*). However, in the groups treated with IL-6 (Fig. 5*B, E*) or glucose (Fig. 5*A, D*) there were fewer than 10% BrdU-positive cells. The results for each group were similar on day 3 (data not shown). Thus, as we had predicted, the therapeutic capacity of hyper-IL-6 is its potential to salvage from death animals suffering from FHF.

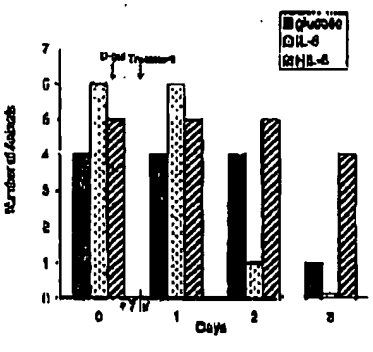

Figure 4. Hyper-IL-6 improves the survival of female rats treated with D-gal to induce lethal FHF. FHF was induced in 15 female Fischer rats (body weight 150–170 g) by single i.p. injections of D-gal at a lethal dosage of 900 mg/kg body weight. Seven hours later they were divided into three treatment groups of four, six, and five rats and treated as described in the legend to Fig. 2.

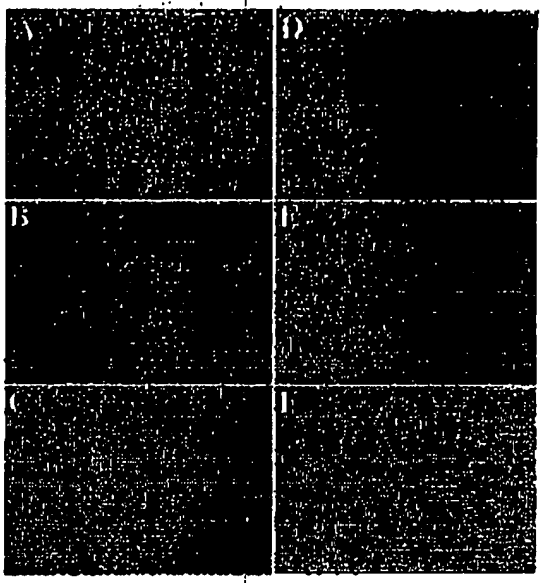

Figure 5. The effect of hyper-IL-6 on hepatocyte proliferation in the livers of female Fischer rats with FHF, as assessed by immunohistological staining for BrdU. Severe hepatotoxicity was induced in female Fischer rats by i.p. injection of D-gal. Seven hours later, the rats were divided into three treatment groups (3–4 animals per group) and treated with 10% glucose (*A, D*), IL-6 (*B, E*), or hyper-IL-6 (*C, F*). Panels *A–C* are low-power images; panels *D, E,* and *F* are high-power images of panels *A, B,* and *C,* respectively. Two days after the administration of D-gal, the rats were pulsed with BrdU; the rats were then killed and the livers were harvested and prepared for microscopy as described in Materials and Methods. The number of positive BrdU cells in low-power field *A* was less than 5%; in panel *B* it was less than 10% and in panel *C* it was greater than 40%.

The early effect of hyper-IL-6 in D-gal-induced FHF

In our initial experiments we observed the effects of hyper-IL-6 on D-gal-induced hepatotoxicity (Fig. 2) during the first 40 h after D-gal injection. We were interested in defining the earliest point at which the physiological effects of treatment by hyper-IL-6 would commence. Severe hepatotoxicity was induced with D-gal, and treatments with hyper-IL-6, IL-6, or glucose were administered according to the protocol described above. In animals treated with hyper-IL-6 5 h after hyper-IL-6 treatment (that is, 12 h after D-gal administration), the activity of factor V started to increase (Fig. 6*A*). At the end of the first day, the activity levels of both coagulation factors V and VII became normalized (Fig. 6*A, B*). In contrast, at the end of the first day the activities of the coagulation factors V and VII in both control groups remained very low. This suggests that the therapeutic effect of hyper-IL-6 starts shortly after administra-

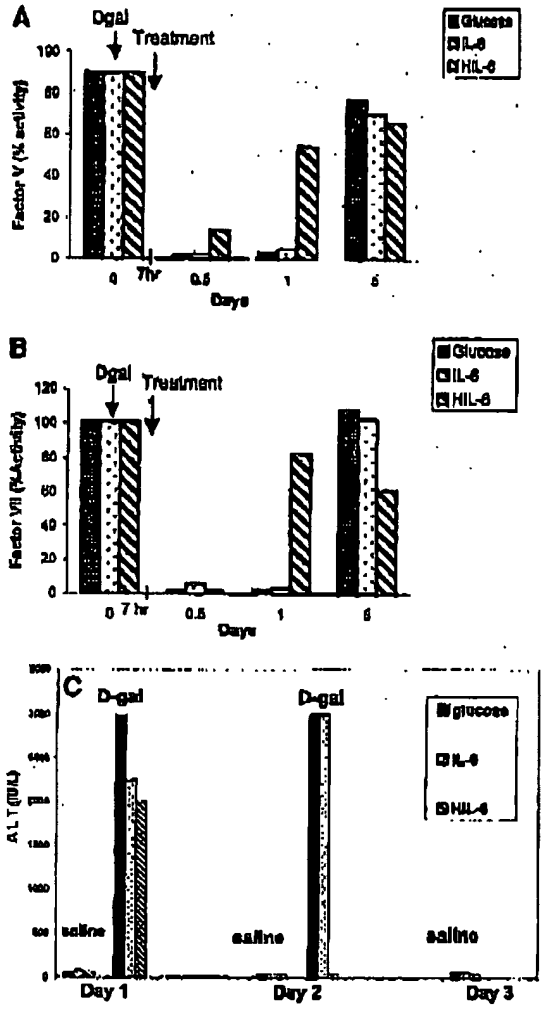

Figure 6. The short-term effects of hyper-IL-6 on severe nonlethal D-gal-induced hepatotoxicity in male rats. The experiment was conducted as described in the legend to Fig. 2 except that there were 16 to 20 rats in each treatment group. At each time point, four to five rats from each treatment group were killed and their blood was used to analyze the activities of coagulation factor V (*A*) and factor VII (*B*). (*C*) Saline neither caused hepatotoxicity nor increased cell proliferation. Three D-gal and three saline administered groups (5 and 8 rats in each group, respectively) were treated with glucose, IL-6, and HIL-6 as described for Fig. 2.

tion, which could have significant ramifications clinical applications.

We further evaluated the potential of HIL-6 to reverse the hepatotoxicity effect of D-gal in the short term. We observed increased ALT levels (Fig. 6C) in all the groups to which D-gal was administered. However, this effect was reversed 24 h later if the animals were treated with HIL-6. On day 3, histological examination of livers of the glucose- and IL-6-treated animals (data not shown) revealed severe liver pathology similar to the changes shown in Fig. 7*A, B*.

As an additional control, we compared animals treated with D-gal to those treated with saline, then 7 h later with glucose, IL-6, or HIL-6. In these saline-treated rats, we observed no increase in the levels of ALT (Fig. 6C) or bilirubin (data not shown) nor did our histological examination reveal signs of liver dysfunction (data not shown). Two days after treatment with saline instead of D-gal, the level of BrdU incorporation in all three groups was the same and observed in less than 1% of the cells (data not shown).

Hyper-IL-6 rescues rats suffering from TAA-induced FHF

Rats in which FHF was induced by TAA were divided into three treatment groups of 10 each. Thirty hours after the induction of FHF, each group was treated i.p. with 5 ml 10% glucose, IL-6 (80 μg/animal), or hyper-IL-6 (8 μg/animal). Note that the amount of hyper-IL-6 administered per animal was 10-fold less than the amount of IL-6 administered per animal. On day 4 after induction of FHF, 1 of the 10 animals treated with glucose survived, 2 of the 10 treated with IL-6 survived, and 4 of the 10 treated with HIL-6 survived (data not shown). These results further support the hypothesis that treatment by hyper-IL-6 is preferable to treatment by IL-6.

The effect of HIL-6 on D-gal-induced apoptosis

D-gal is known to induce significant apoptosis, although the exact mechanism for this effect is not known (22). As expected, we found that 2 days after rats were treated with HIL-6 after the induction of severe hepatotoxicity, there was a significantly lower level of apoptotic cells than when the animals were treated with IL-6 (Fig. 8) or glucose (data not shown).

DISCUSSION

Terminally differentiated hepatocytes can undergo sustained cell proliferation. Overturf and colleagues (29) have shown that in the hereditary tyrosinemia type I model, fewer than 10,000 hepatocytes can repopulate a complete liver. This process of liver regeneration is enhanced by several cytokines, particularly by IL-6 (7). IL-6 exerts its effect through its interaction with one of its receptors: IL-6Rα. Together they form a complex that induces the homodimerization of two gp130 signal transduction molecules (20, 24). The soluble form of IL-6Rα

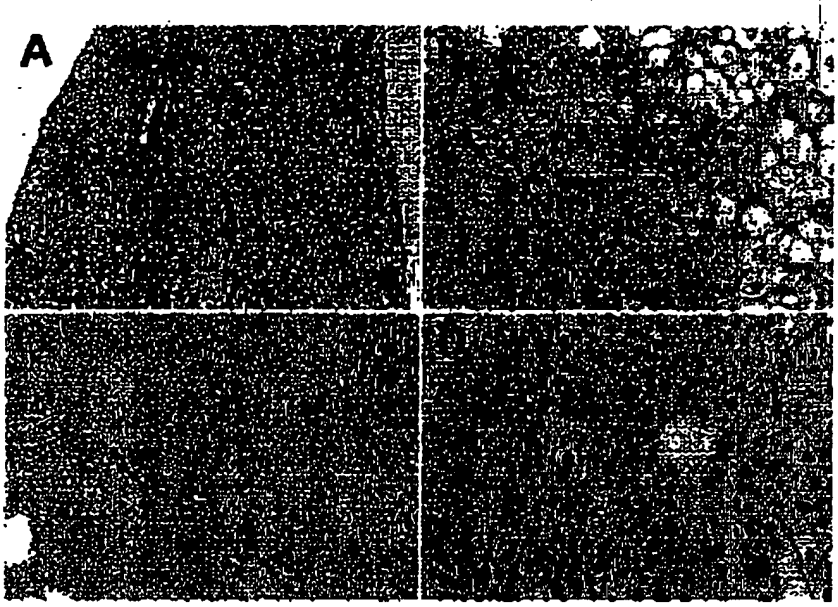

Figure 7. Liver histology of rats 3 days after D-gal administration and subsequent treatment with glucose (*A*, low-power; *B*, high-power) or hyper-IL-6 (*C*, low-power; *D*, high-power). Note the normal architecture with focal macrovesicular steatosis and Kupffer cell hyperplasia in the liver of the hyper-IL-6-treated animal. In contrast, the livers of the glucose or IL-6 (not shown) -treated animals showed marked cholangiolar proliferation, mild inflammation, macrovesicular steatosis, and ballooning degeneration.

(sIL-6R) causes IL-6Rα-/gp130+ cells to be responsive to IL-6 (25). In both TNF-RI- and IL-6-deficient mice, liver regeneration is significantly impaired (11, 26). In both cases liver regeneration could be restored to normal by treatment with either IL-6 or with the IL-6/sIL-6R complex. An explanation for the effect of the IL-6/sIL-6R complex can be based on the results of experiments on mice singly transgenic for IL-6 alone or doubly transgenic for both IL-6 and sIL-6R. It is possible that this treatment bypasses the need for NF-κB, suggesting a role for GP130 signaling in liver regeneration (9, 27, 28). An explanation for the effect of the IL-6/sIL-6R complex comes from experiments with mice either transgenic for IL-6 or doubly transgenic for both IL-6 and sIL-6R. In doubly transgenic sIL-6R/IL-6 mice, the sIL-6R molecule acts as an anchor for IL-6, resulting in a prolonged plasma half-life for IL-6. The cells also become dramatically sensitized to IL-6 (18). The presence of the IL-6/sIL-6R complex in doubly trans-

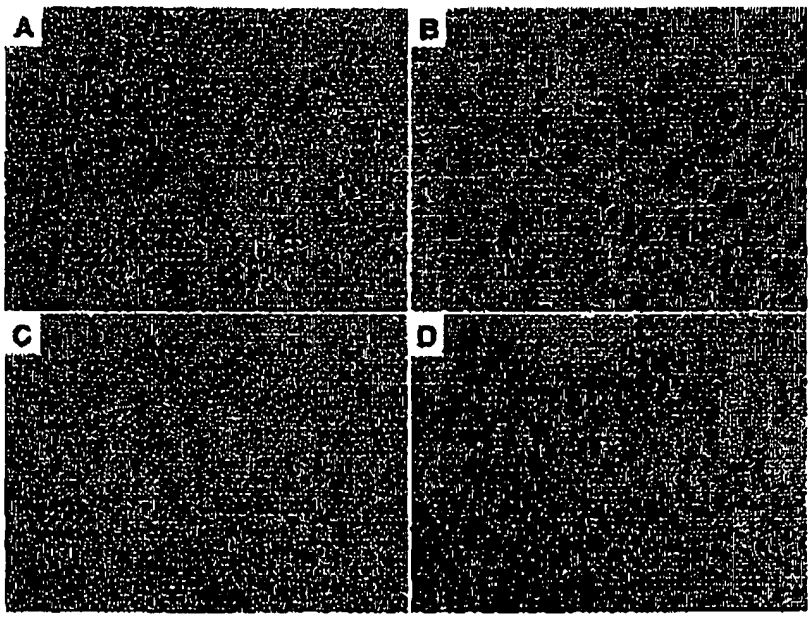

Figure 8. HIL-6 reduces liver cell apoptosis in male rats with FHF induced by D-gal. Liver injury was induced in rats by D-gal; 7 h later, the rats were treated with IL-6 or HIL-6. Two days later, the livers of the treated animals were harvested and assessed for apoptosis by the TUNEL assay. In the livers of IL-6-treated rats, more than 80% of hepatocyte nuclei were apoptotic (*A*, low-power; *B*, high-power). In the livers of HIL-6-treated rats, fewer than 20% of hepatocyte nuclei were apoptotic (brown staining) (*C*, low-power; *D*, high-power).

genic mice also has a marked effect on hepatocyte proliferation, which is not observed in mice transgenic for IL-6 alone (18, 19). These findings prompted us to develop a designer cytokine, which we have called hyper-IL-6, that consists of IL-6 covalently linked to the sIL-6R through a flexible amino acid linker. We found hyper-IL-6 to be 100- to 1000-fold more active than the combination of the separate proteins IL-6 and sIL-6R (20, 21).

Here we report on the *in vivo* ability of the hyper-IL-6 cytokine to reverse both sublethal and lethal hepatotoxic damage induced by D-gal in rats. In our experiments, neither glucose nor IL-6 could replace hyper-IL-6. When the D-gal-induced liver damage was sublethal, liver cells started to proliferate shortly after treatment with hyper-IL-6 and the toxic injury was reversed in less than 24 h (Fig. 6). The recovery in rats treated with IL-6 or glucose occurred simultaneously 24 to 48 h later, suggesting that this delayed event was spontaneous, possibly related to endogenous generation of IL-6 and sIL-6R or a panel of cytokines and growth factors. Note, however, that 10-fold more IL-6 (80 μg) was used than hyper-IL-6 (8 μg). Moreover, we found only minor pathological alterations the liver histology of the hyper-IL-6-treated group, whereas the livers of animals in the two control groups revealed severe liver damage. These changes persisted in the livers of the IL-6- and glucose-treated rats and were detected on day 3 (Fig. 7*A*, *B*). In contrast, at the same time we found only minor pathological alterations in the hyper-IL-6-treated animals (Fig. *C*, *D*), suggesting that the therapeutic effect of hyper-IL-6 is achieved through enhanced hepatocyte proliferation. This early recovery of the hyper-IL-6-treated group we observed histologically coincided with a complete recovery of hepatocyte synthetic functions, as apparent from the serum activity levels of coagulation factors V and VII and normalization of the levels of serum bilirubin, glucose, and ALT (Figs. 2 and 6). Furthermore, in all three of our stringent models, treatment with hyper-IL-6 significantly improved the survival rate of rats with D-gal-induced FHF.

Although at this time we can only speculate on the mechanism by which hyper-IL-6 reverses liver toxicity, we propose a hypothetical algorithm for this effect: D-gal might induce apoptosis by sensitizing the liver cells to TNF-α (29, 30). The designer cytokine hyper-IL-6 is a potent activator of the signal transducer and an activator of transcription STAT3 (31). Both *in vitro* and *in vivo*, hyper-IL-6 is more than 10-fold more effective than IL-6 in stimulating STAT3-dependent gene transcription in liver cells. STAT3 is known to be involved in liver regeneration and is also anti-apoptotic (32, 33). Together, these facts strongly suggest that the activating the gp130 signal transduction pathway may induce enhanced cell proliferation. In fact, we observed that HIL-6 did have a strong anti-apoptotic effect (Fig. 8). It is possible that this effect might be induced by HIL-6 gp130 signaling the activation of STAT-3 (34). Thus, based on these ideas and on the results that we have reported here, we propose that hyper-IL-6 has an anti-apoptotic effect that reverses the D-gal hepatotoxicity and enhances liver regeneration. It is also known that patients with liver damage have increased serum IL-6. IL-6 stimulates the production of acute-phase proteins (e.g., haptoglobin), and these proteins have recently been found to induce the shedding of IL-6R from macrophages (35). The resulting increase in blood levels of sIL-6R would thereby be expected to enhance the direct effect of IL-6 on liver regeneration.

We have shown that the fusion protein hyper-IL-6 has a marked stimulating effect on liver regeneration in rat FHF and severe hepatotoxicity models. Our results point both to an important role for sIL-6R during liver regeneration and suggest that hyper-IL-6 has a significant therapeutic potential whereby it could be used to reverse the state of FHF or to enhance liver regeneration in various clinical conditions related to acute or chronic liver damage.

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ARTICLE

Hyper-IL-6 Gene Therapy Rev rses Fulminant Hepatic Failure

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Fulminant hepatic failure is a catastrophic condition caused by massive hepatocellular apoptosis and necrosis. Inhibition of hepatocyte apoptosis and the enhancement of the endogenous potential for liver regeneration could potentially form an effective basis for treatment of this condition. In response to injury in the liver, IL-6 mediates the acute-phase response and induces both cytoprotective and mitogenic functions. Hyper-IL-6 is a superagonistic designer cytokine consisting of human IL-6 linked by a flexible peptide chain to the secreted form of the IL-6 receptor. In a mouse model of acute liver failure induced by D-galactosamine administration, a single low dose of a hyper-IL-6-encoding adenoviral vector, in contrast to an adeno-IL-6 vector, maintained liver function, prevented the progression of liver necrosis, and induced liver regeneration, leading to dramatically enhanced survival. Thus, hyper-IL-6 gene therapy may be useful for the treatment of fulminant hepatic failure, which is often fatal even following treatment by transplantation.

Key Words: **fulminant hepatic failure; gene therapy; hyper-IL-6; IL-6/sIL-6R; gp130 hyperstimulation; D-galactosamine.**

INTRODUCTION

Fulminant hepatic failure (FHF) remains one of the more challenging conditions in clinical medicine today (1). It is caused by massive hepatocellular apoptosis and necrosis that can be induced by many factors, including viral hepatitis, chemical- and drug-related toxicity, and metabolic disorders (2–4). As a result of the lack of effective therapies and due to various complications, the mortality rate from FHF is high, exceeding 60% (1, 2). Inhibition of hepatocyte apoptosis and the enhancement of the endogenous potential for liver regeneration could potentially be an effective basis for the treatment of FHF (5, 6).

Following injury, the liver has a remarkable capacity to restore major tissue loss through regeneration (6). The molecular signaling mediating hepatocyte proliferation during liver regeneration is a complex process involving priming factors, including tumor necrosis factor-α and interleukin-6 (IL-6), and a number of growth factors, of which hepatocyte growth factor and transforming growth factor-α appear to be most important (6).

IL-6 is a pleiotropic cytokine (7), which mediates the acute-phase response in the liver and has both cytoprotective and mitogenic functions (6). IL-6 is a member of a family of cytokines that act via receptor complexes containing at least one subunit of the transmembrane signal transducing protein gp130 (8). On target cells, IL-6 acts by binding to a specific transmembrane cognate receptor (gp80 or IL-6R), which triggers the homodimerization of gp130 and leads to the activation of the Jak/Stat signaling pathway (8, 9). Hyper-IL-6 is a superagonistic designer cytokine consisting of human IL-6 linked by a flexible peptide chain to a soluble form of the IL-6 receptor (sIL-6R) (10, 11).

In this study we have explored the potential of hyper-IL-6 gene therapy using adenoviral vectors to maintain liver function, prevent the progression of liver necrosis, and induce liver regeneration as a treatment for FHF.

MATERIALS AND METHODS

Animals. Male C57Bl/6 mice (Harlan Animal Breeding Center, Jerusalem) age 4–5 weeks were used. Acute liver failure was induced by intraperitoneal administration of 25–30 mg per mouse of a D-galactosamine (D-gal) (Sigma) solution, prepared in 0.89% saline adjusted to pH 6.8 with 1.0 N NaOH, to 12-h-fasted mice. Following D-gal administration mice were kept under fasting conditions for an additional 12 h, but provided with water containing 10% glucose ad libitum in order to maintain glucose levels. Treatment of mice with viruses or control solutions, in a volume of 0.1 ml, was performed by intraperitoneal injection 7 h post-D-gal administration. Serum samples were obtained from treated mice by bleeding from the tail

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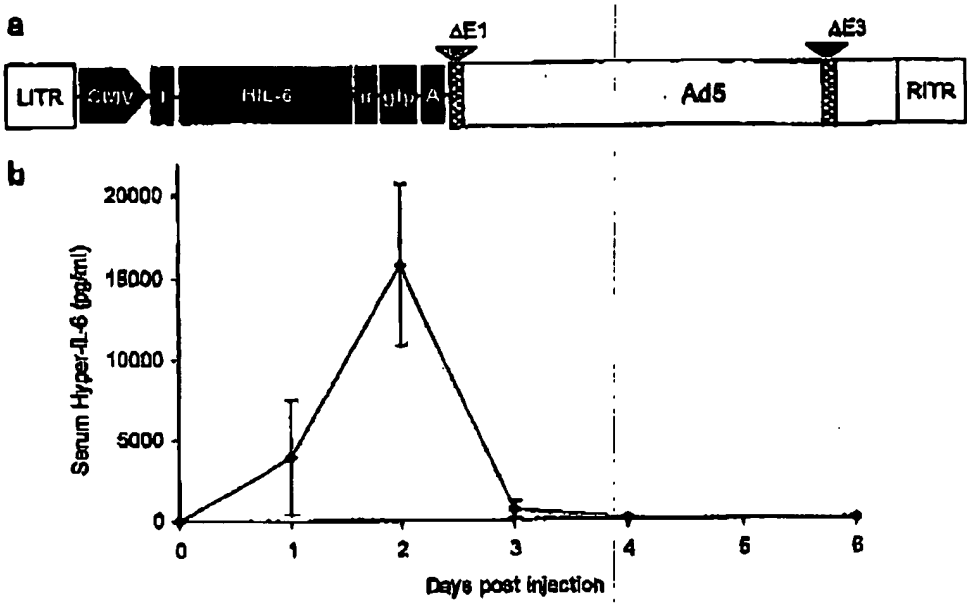

FIG. 1. Adenoviral vector Ad.HIL6gfp. (a) The recombinant adenoviral vector Ad.HIL6gfp contains the CMV immediate-early enhancer/promoter (CMV), a chimeric intron (I), and a bicistronic gene containing the 1.6-kb hyper-IL-6 (HIL-6) cDNA, followed by the poliovirus IRES (ir) and an enhanced green fluorescence protein gene (gfp), followed by the late SV40 polyadenylation site in an E1/E3-deleted Ad5 backbone (12). (b) Serum hyper-IL-6 levels in normal mice (n = 10) that received a single dose (ip) of 10^8 TU Ad.HIL6gfp. Values are means ± standard error of mean.

vein. In experiments involving serum analysis of the animals following induction of liver damage, separate sets of animals were sampled on the days indicated.

Adenoviral vector construction and preparation. Adenoviral vectors were constructed using the AdEasy system (12). Ad.HIL6gfp was constructed using a human hyper-IL-6 cDNA gene coding the human sIL-6R (amino acid residues 1-323) and human IL-6 (amino acid residues 29-212) fused by a synthetic DNA linker coding for the amino acid sequence Arg-Gly-Gly-Gly Ser Gly-Gly-Gly-Gly-Ser-Val-Glu (10). In order to allow convenient monitoring of adenoviral transduction and gene expression, a bicistronic hyper-IL-6/IRES (poliovirus)/gfp gene construct was made by ligation of a *SnaBI–NotI* DNA fragment containing a portion of the CMV promoter and the hyper-IL-6 gene derived from the expression plasmid pCI-HIL6 to the same sites in the plasmid pCEMIRESegfp (a kind gift from W. Lindenmaier, GBF, Braunschweig, Germany). To prepare the Ad.HIL6gfp virus construct, an *AclI–NruI* DNA fragment from pCEMhIIGIRESgfp containing the CMV promoter-driven HIL6gfp gene construct was cloned into the *EcoRV* site of pShuttle and subsequently introduced into the pAdEasy-1 vector by homologous recombination in BJ5183 cells, as described (12). Ad.HIL6gfp was rescued from pAdEasy-HIL6gfp by transfection in 293 cells and plaque purified twice by limiting dilution. The Ad.IL6gfp virus was constructed in a similar manner using the human IL-6 cDNA. Vector-mediated transgene expression was confirmed by analysis of conditioned media from adenoviral vector-transduced 293 or HUH-7 cells on BAF/3/gp130 and BAF/3/gp130/IL-6R indicator cell lines (10), which are dependent on hyper-IL-6 and IL-6, respectively, for cell proliferation. The control virus, Ad.gfp, was kindly provided by H. Giladi (Hadassah Medical Organization) and was constructed by homologous recombination of pAdEasy-1 with the pAd-Track shuttle vector (12). For large-scale adenoviral preparations, 293 cells cultured on 15-cm dishes in DMEM supplemented with 10% fetal calf serum and 2 mM glutamine were infected at a multiplicity of infection of 1-5 and grown for 2-3 days. The infected cells were then collected by centrifugation (2000*g* for 5 min) and viruses harvested by gentle lysis (three cycles of freeze/thawing) of cells in a solution of PBS containing 0.68 mM $CaCl_2$, 0.50 mM $MgCl_2$, and 10% glycerol. The cell extracts were then clarified by centrifugation at 10,000*g* for 4 min at room temperature and stored at −80°C for use in animal studies. Viral titers were determined by infection of 293 cells cultured in 24-well dishes and scored 1-2 days after infection by visualization of the gfp marker using an inverted fluorescence microscope.

Serum biochemistry and IL-6 enzyme-linked immunosorbent assay (ELISA). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using a Reflotron (Roche) and Reflotron test strip reagents. Normal calf serum was used to dilute samples with transaminase levels in excess of the assay linear range. Hyper-IL-6 and human IL-6 levels in mouse serum were determined using a Pelikine Compact human IL-6 ELISA kit (CLB, Amsterdam), which is specific for human IL-6.

Histological and immunohistochemical examination. Paraffin-embedded sections were stained with hematoxylin-eosin. For immunohistochemical staining, sections were incubated in 0.01 M citrate buffer, pH 6.0, and heated by microwave (750 W) to boiling for 2 min and cooked at 20% full power for a further 20 min. The samples were left in the heated buffer for 20 min, then rinsed with distilled water and blocked with a solution of 1% BSA in PBS containing 0.5% Triton X-100. The sections were stained with an anti-PCNA (PC10) mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and detected using the EnVision+ HRP,Mouse (DAKO, Copenhagen) kit developed with 3-amino-9-ethylcarbazole and counterstained with hemarmaytin.

Results and Discussion

An Adenoviral-Based Gene Therapy Strategy for Transient Hyper-IL-6 Delivery in Vivo

After a single intraperitoneal injection of a small dose (10^8 transducing units, TU) of the adenoviral vector Ad.HIL6gfp (Fig. 1a), into normal, otherwise untreated C57Bl/6 mice, a substantial but transient expression of the designer cytokine could be detected by human IL-6 ELISA. Hyper-IL-6 serum levels reached a peak of approximately 20 ng/ml 2 days following injection and rapidly diminished to undetectable levels by day 6 (Fig. 1b). Serum levels of the Ad.HIL6gfp-directed hyper-IL-6 protein varied significantly between individual subjects, but the same pattern of expression was observed repeatedly in numerous experiments. At this dose, the level of hyper-IL-6 expression did not produce any apparent morbidity in the mice. However, a dose of 10^9 TU produced notable morbidity, as manifest by the cessation of eating and

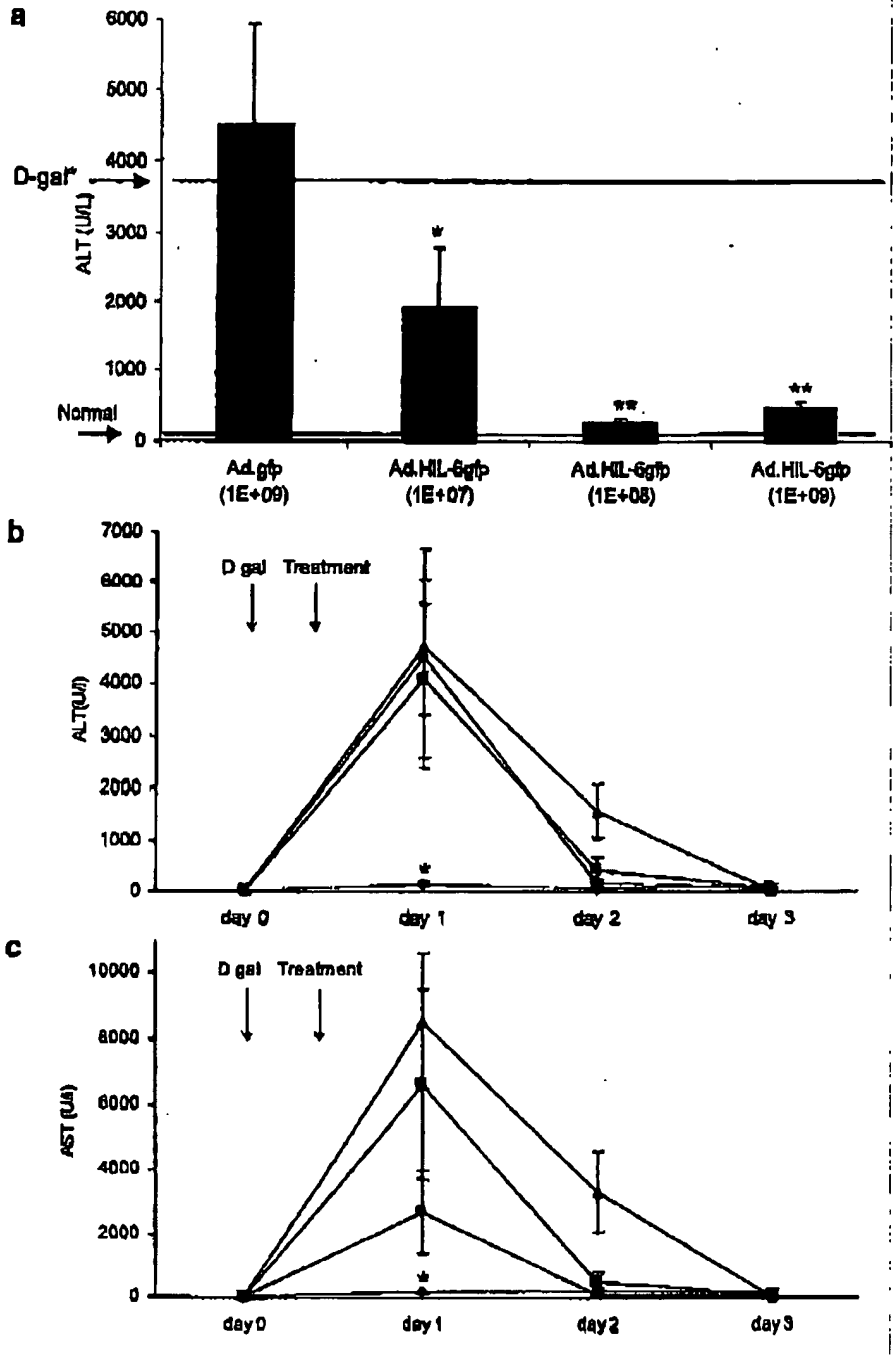

FIG. 2. Ad.HIL6gfp reduces the appearance of D-gal-induced acute hepatitis in mice. (a) Dose-response analysis. Serum ALT levels in mice (n = 7) 1 day following induction of liver damage with a sublethal dose of D-gal (25 mg) and treatment 7 h later with varying doses of Ad.HIL6gfp or the control vector, Ad.gfp. The average ALT serum level in control D-gal-administered mice treated with PBS, as derived from two separate experiments, is shown (D-gal*, ALT = 3627 ± 1336 U/L; n = 14). Normal, ALT level in normal untreated mice. *$P < 0.1$ and **$P < 0.015$, compared with Ad.gfp, Student's t test. (b and c) Comparison of serum ALT (b) and AST (c) levels in mice (n = 8 per day) following induction of liver damage with a sublethal dose of D-gal and treatment 7 h later with 10^8 TU Ad.HIL6gfp (◆), Ad.IL-6 (■), Ad.gfp (▲), or PBS (●). Day 0 represents baseline values found in normal mice before treatment with D-gal. Values are means ± SEM. *$P < 0.006$ compared with Ad.gfp-treated mice. Student's t test.

grooming behavior and assumption of immobile hunched postures, followed by mortality that reached 100% in some experiments. An identical dose of Ad.gfp produced no overt signs of illness in the animals. The cause of death of the mice that received the highest dose of Ad.HIL6gfp is not clear. However, no evidence of liver damage was found in either moribund or deceased animals, as determined by both histological examination of liver sections and analysis of serum transaminase levels.

Ad.HIL6 Gene Therapy Ameliorates D-Galactosamine-Induced Acute Hepatic Failure

The liver-specific toxin D-galactosamine causes focal and diffuse hepatocyte apoptosis followed by necrosis,

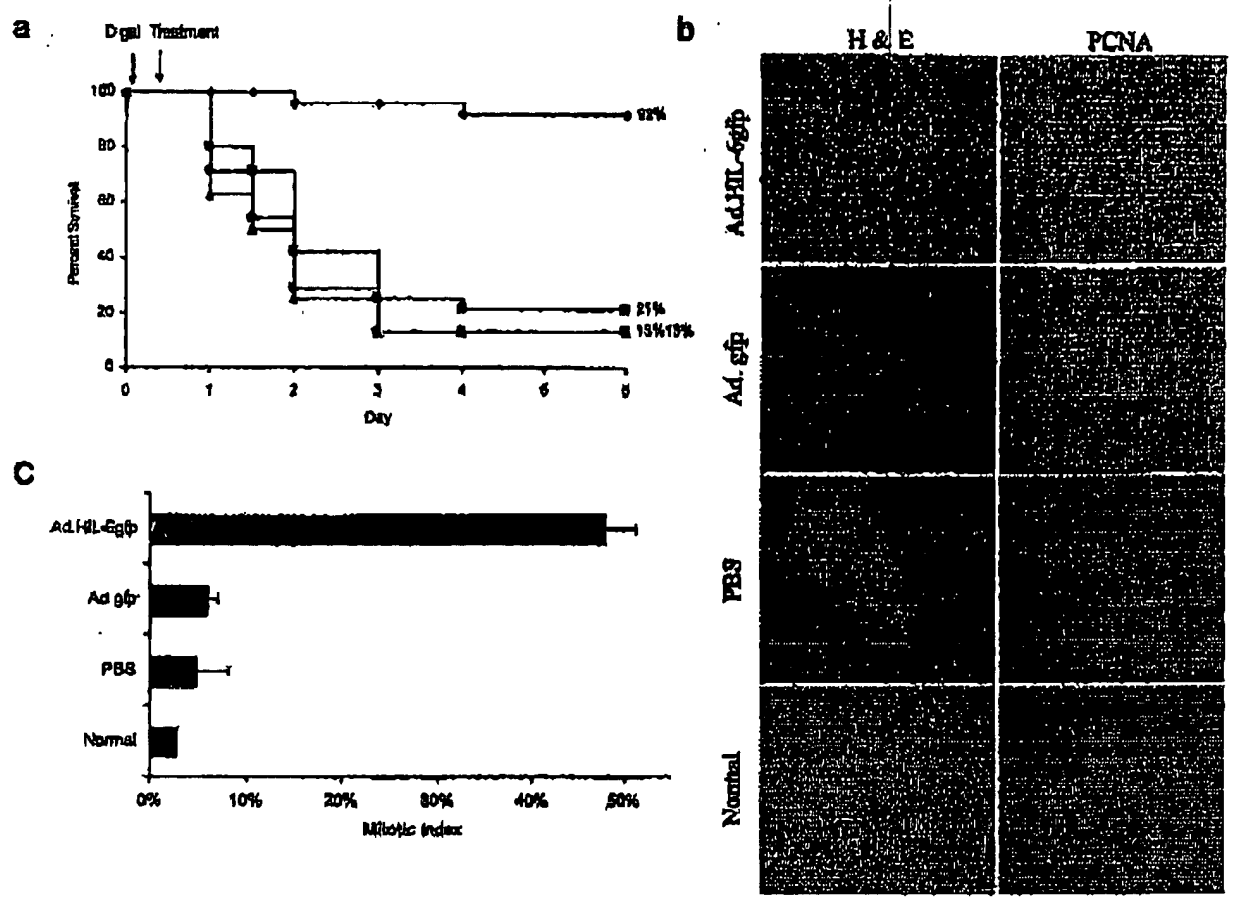

FIG. 3. Liver regeneration and enhanced survival of FHF following treatment with Ad.HIL6gfp. (a) Kaplan–Meier plot of survival following a lethal dose of D-gal (30 mg) in male C57BL/6 mice ($n = 24$) and treatment with 10^{9} TU Ad.HIL6gfp (◆), Ad.IL-6 (■), Ad.gfp (▲), or PBS (●). (b) Histopathological analysis. Livers from animals administered a lethal dose of D-gal (30 mg) and treated with 10^{8} TU Ad.HIL6gfp, 10^{8} TU Ad.gfp, or PBS were removed 1 day after treatment. Normal, untreated mouse liver. Liver sections were stained with hematoxylin–eosin (H&E, left) or with anti-PCNA antibody (PCNA, right). Original magnification ×200. (c) Histogram of percentage PCNA-positive hepatocytes in liver sections from mice as in b. Values are presented as means ± SEM of 2000 nuclei counted ($n = 2$, Normal liver $n = 1$).

infiltration of polymorphonuclear cells, enlargement of macrophages resembling drug-induced hepatitis, and hepatic failure (13–15). Treatment of mice with a sublethal dose of D-gal (25 mg) induces substantial liver damage that is manifest by a transient increase in serum levels of liver-related enzymes (Fig. 2). The effect of the D-gal is rapid, and liver damage is clearly observable within several hours following D-gal administration (15–17). Elevated serum levels of liver transaminases reach a peak on the first day following D-gal treatment, but decrease thereafter and are completely resolved by day 3 in surviving animals. In order to determine whether a liver-directed gene therapy strategy can maintain liver function and arrest progressing liver necrosis, we performed a dose–response analysis using from 10^{8} to 10^{0} TU of the Ad.HIL6gfp vector given 7 h post-D-gal administration. The results of this analysis revealed that doses of 10^{8}–10^{0} TU were almost equally effective in treatment of liver injury (Fig. 2a) as manifest by the reduced serum transaminase levels in comparison to the D-gal-treated control. A dose of 10^{7} TU was less effective in preventing the appearance of liver injury and a dose of 10^{6} TU was ineffective (data not shown). However, in contrast to its effect on normal mice, a dose of 10^{9} TU of the Ad.HIL6gfp vector in the D-gal-treated animals was neither lethal nor notably toxic. When followed over a period of several days (Figs. 2b and 2c), mice that were treated with the optimal dose of Ad.HIL6gfp (10^{8} TU) displayed only mild liver injury, which was significantly less than all other groups. Mice treated with the control adenoviral vectors, Ad.gfp (10^{8} TU), or Ad.IL6gfp (10^{9} TU) displayed the typical pattern of liver injury similar to that of the PBS-treated control mice. In several experiments, it appeared that the mice treated with Ad.gfp had even greater liver injury, which might be expected from the reported cytopathic effect of adenoviral vectors (18, 19). But this effect was not significant ($P > 0.35$).

Treatment with Ad.HIL6gfp Reduces Lethality after D-Gal-Induced FHF

Within less than 1 day following treatment with a lethal dose of D-gal (30 mg/mouse), mice develop apoptosis and diffuse necrosis of the liver parenchyma (15, 17) that

rapidly progresses to FHF and death (Fig. 3a). Few of the PBS control-treated mice survived to day 7 following b-gal administration (Fig. 3a) and mice treated with the control adenoviral vector, Ad.gfp, faired equally poorly. Histopathological analysis of livers taken from PBS-treated and Ad.gfp-treated mice that remained alive 24 h following a lethal dose of b-gal revealed focal to extensive necrosis accompanied by infiltration of polymorphonuclear cells (Fig. 3b), explaining the rapid deterioration and death of the animals. Liver sections from mice treated with Ad.IL6gfp had an appearance similar to that of the PBS-treated controls, but nevertheless had consistently higher survival rates, suggesting that despite the poor histopathological profile the IL-6 vector indeed provided a small therapeutic effect. In comparison, mice treated with Ad.HIL6gfp maintained a fairly normal liver morphology with little or no inflammation and only occasional apoptotic bodies. Almost all of the Ad.HIL6gfp-treated animals recovered from the lethal effects of the b-gal by day 7. All mice that survived to day 7 remained alive for the duration of the 3-week observation period following treatment. To determine whether the maintenance of liver function and survival of the Ad.HIL6gfp-treated mice were associated with hepatocyte proliferation we performed immunohistochemical staining for proliferating cell nuclear antigen (PCNA) to assess the fraction of hepatocytes that are in the S phase of the cell cycle 1 day following treatment (Fig. 3b). In Ad.HIL6gfp-treated mice, the number of PCNA-positive hepatocytes was dramatically higher than in either the Ad.gfp-treated mice or the PBS-treated mice. The labeling index analysis showed that about 50% of the hepatocytes were in mitotic cycle in the Ad.HIL6gfp-treated mice at 24 h post-treatment, compared to 8% in control groups and about 3% in normal mouse liver (Fig. 3c).

Here we have demonstrated that a single low-dose administration of a recombinant adenoviral vector carrying the designer cytokine gene hyper-IL-6 inhibited apoptosis and necrosis and stimulated hepatocyte proliferation, resulting in maintenance of liver function and complete recovery from fulminant hepatic failure. Systemic administration of adenoviral vectors overwhelmingly favors transduction of liver (20). In this study, we have taken advantage of this empirical preference of hepatocyte transduction with the aim of increasing local hyper-IL-6 concentrations in the liver parenchyma. In a strategy of this design, the efficiency of vector transduction will depend on the amount of healthy tissue available for vector transduction. In this study we have observed that despite the presence of active liver disease progression in the treated animals at the time of vector administration, transgene expression was significant and effective. This observation, in agreement with that of Nakatini *et al.* (21), supports the notion that transient or regulated expression of a gene therapy delivered product targeted to the liver can serve as a therapeutic strategy even in the presence of severe, progressing liver disease. Whether alternative routes of vector administration, such as intramuscular injection, can be equally effective in the treatment of FHF will be addressed in future studies. In conclusion, the results of this study demonstrate the therapeutic potential of hyper-IL-6 to treat FHF and reinforce the concept that stimulation of hepatocyte regeneration can serve as a therapeutic strategy to obviate the necessity of liver transplantation and enhance survival from an otherwise fatal condition.

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